

Abstract

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- ### Short Communications

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Possible role of thiamine in neuromuscular transmission

By

LENNART WALDENLIND

Received 28 November 1977

Abstract

WALDENLIND, L. Possible role of thiamine in neuromuscular transmission. *Acta physiol. scand.* 1979 105: 1-10.

Pyridoxamine (50 mg/kg), thiamine antagonist, decreased the muscle twitches of the rat masseter muscle at stimulation frequencies above 1 Hz 40-80 min after an i.v. injection. The post-tetanic potentiation (PTP) induced by tetanic stimulation of the masseter muscle is abolished by pyridoxamine. Administration of thiamine restored the muscle twitches at stimulation frequencies above 1 Hz and the PTP. The muscle twitches elicited by direct muscle stimulation were not affected by pyridoxamine treatment. The abolishment of the PTP is accompanied by decrease in thiamine and thiamine-diphosphate. The pyruvate level in the blood is unchanged after pyridoxamine treatment. Oxythiamine, on the other hand, had no effect on the PTP but increased the pyruvate level in the blood. Fern extract, which contains thiaminase I also abolished the PTP—an effect reversible by the addition of thiamine. The frequency-induced depression of the muscle twitches induced by pyridoxamine is similar to the effect of low doses of d-tubocurarine (4 μ g/kg). The results support the hypothesis that thiamine may play a role in neuromuscular transmission.

Thiamine has been described to be of importance for the conduction of nerve impulses (Murah 1947). It was suggested that thiamine participates in the metabolism of the nerve by acting as a metabolic substance and not only as a "catalyst" since more thiamine could be extracted by a Ringer solution from a freeze-dried nerve powder of excited nerves than could be extracted from a corresponding sample of unexcited nerves.

A stimulus-dependent release of thiamine from the curarized phrenic nerve-diaphragm preparation has been described (Waldenlind 1977). To investigate possible role of thiamine in neuromuscular transmission the effect of thiamine on the miniature end plate potentials of the frog sartorius muscle was studied (Waldenlind *et al.* 1978). Thiamine was found to decrease the miniature end plate potentials significantly and reversibly. Thiamine was also found to bind to isolated nicotinic receptors from *Torpedo marmorata* (Waldenlind 1978) supporting the idea that thiamine binds to nicotinic receptors or to closely related structures. However the concentration of thiamine used was rather high ($2 \cdot 10^{-4}$ M).

The present study has been made in order to study if thiamine, in addition to its proposed action on neuroconduction, is of importance for neuromuscular transmission. The effect

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Pyridoxamine (30 mg/kg), thiamine antagonist, decreased the muscle twitches of the rat sartorius muscle at stimulation frequencies above 1 Hz 40-80 min after an i. injection. The post-tetanic potentiation (PTP) induced by nerve stimulation of the sartorius muscle was abolished by pyridoxamine. Administration of thiamine restored the muscle twitches at stimulation frequencies above 1 Hz and the PTP. The muscle twitches elicited by direct muscle stimulation were not affected by pyridoxamine treatment. The abolishment of the PTP was accompanied by decrease in thiamine and thiamine-diphosphate. The pyruvate level in the blood was unchanged after pyridoxamine treatment. Orythiamine, on the other hand, had no effect on the PTP but increased the pyruvate level in the blood. Fern extract, which contains thiaminase I also abolished the PTP—an effect reversible by the addition of thiamine. The frequency-induced depression of the muscle twitches induced by pyridoxamine was similar to the effect of low doses of d-tubocurarine (0.1 mg/kg). The results support the hypothesis that thiamine may play a role in neuromuscular transmission.

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The present study has been made in order to study if thiamine, in addition to its proposed function in neuroconduction, is of importance for neuromuscular transmission. The effect

of thiamine antagonists (pyrithiamine, oxythiamine, fern extract) on the muscle twitches induced by nerve stimulation has been compared with the effect on muscle twitches induced by direct stimulation.

Methods

Male Sprague Dawley rats (300 g) were anesthetized with pentobarbital (30 mg/kg). The trachea was intubated with a plastic tube just below the larynx. The external jugular vein was cannulated to allow intravenous injections. The blood circulation of the masseter muscle was intact. The masseteric nerve was prepared and cut off proximally. Supramaximal stimulation (1.5 times that required for maximal stimulation) of the masseteric nerve was then carried out either directly or by field stimulation (0.2 Hz; 0.2 ms) by a Grass SD 9 stimulator using stainless steel electrodes. The isometric twitches of the masseter muscle were measured by a thread around the teeth connected to a Grass force-displacement transducer (FT 10) and a Grass polygraph (model 7 B). The contraction force registered is proportional to the tension in the masseter muscle. The initial tension was equivalent to 150 g. The contraction force is given in the individual figures. The PTP was provoked by a brief tetanic stimulation at 30 Hz or at 50 Hz for 5 s. Since the untreated muscle responded with individual twitches at frequencies at 30 Hz (typical for fast muscle) the term PTP is somewhat incorrect. However it is an accepted term for the phenomenon studied and therefore a new term as increase in muscle twitches after stimulation at 30 Hz was avoided even if this term would have been more correct.

The PTP was calculated by measuring the increase in height of the five first potentiated muscle twitches. The mean value for the increase in heights of the five twitches was thereafter expressed as the ratio between the mean increase of the five muscle twitches and a nonpotentiated twitch.

All substances were injected i.v. in volumes of 0.4 ml (except fern extract where a volume of 2-4 ml was used). All substances were dissolved in 150 mM NaCl and the pH adjusted to 7.3-7.4 with NaOH.

Thiamine determination

Thiamine and its phosphorylated derivatives were measured in biopsies (~100 mg) from the stimulated masseter muscle. The biopsies were homogenized in 0.5 ml of 0.1 M HCl, boiled for 30 min in sealed ampoules and then extracted and measured according to Waldenlind (in press). In order to eliminate pyrithiamine fluorescence for the calculations, an aliquot of the sample was treated with 15% KOH for 60 min before addition of the ferricyanide to destroy the thiamine. The thiamine content could then be measured by comparing the fluorescence in the samples which were treated with KOH with those that were not.

Materials

Caffeic acid, d-tubocurarine, oxythiamine, pyrithiamine and thiamine were obtained from Sigma Chemical Co.

Results

Effects of pyrithiamine on the PTP

Pyrithiamine is a thiamine antagonist that on administration to animals induces the same neurological symptoms as thiamine deficiency (Stein-Parvé 1967). It has little effect on the coenzyme functions of thiamine diphosphate (Th-DP) (Stein-Parvé 1967).

Administration of pyrithiamine (50 mg/kg) had no immediate effect on the PTP. After 40-80 min, however, the PTP disappeared whereas the muscle twitches elicited by low frequency stimulation were unchanged (Fig. 1) (Table 1). No difference was seen if the tetanic stimulation was carried out at 30 Hz for 5 s or at 50 Hz for 20 s. It can also be seen in Fig. 1 that the muscle twitches during the high frequency stimulation were depressed. Direct stimulation of the muscle fibres, however, gave rise to PTP (Fig. 1) (n=3). Thus the effect of pyrithiamine is on neurotransmission and not on the muscle itself. Addition of

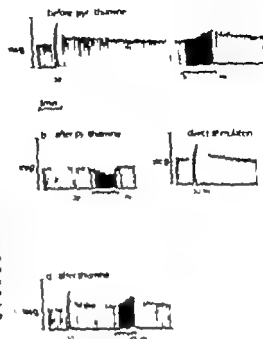


Fig. 1. Effect of pyridoxamine on PTP and muscle twitches at high frequency stimulation. The single muscle twitches were evoked by stimulating at 0.2 Hz and the PTP as evoked by stimulating at 30 Hz for 5 s. The start-cess effect as evoked by continuous stimulation at 0.5, 1.0, 2.0, 4.0 and 8.0 Hz respectively. a) before injection of pyridoxamine, b) 25 min after injection of pyridoxamine (20 mg/kg), c) direct muscle stimulation after pyridoxamine administration, the PTP induced by nerve stimulation was abolished, d) 5 min after the injection of thiamine (20 mg/kg) PTP reappeared as did the muscle twitches at high frequency stimulation.

thiamine (10–20 mg/kg) restored the PTP in a few minutes—an effect that lasted at least 60 min. Addition of Tb-DP (20 mg/kg) had no restoring effect. In control experiments ($n = 4$) where no thiamine was given, the PTP did not reappear. Instead the ordinary twitches began to decrease gradually 15–25 min after the loss of the PTP. At this stage no recovery of the PTP was achieved with thiamine.

Effect of oxythiamine on the PTP

Oxythiamine is a thiamine antagonist that inhibits the coenzyme reaction of Tb-DP (Stern-Purvis 1967). It is phosphorylated to oxythiamine diphosphate and inhibits thiamine-dependent reactions by competition with Tb-DP.

In the present experiments, oxythiamine in doses up to 2 g/kg had no significant effect on the PTP even up to 6 h after administration (Fig. 2) (Table I). The oxythiamine had to be injected in small portions (50 mg/kg). Otherwise a reversible and short-acting curare-like action was seen.

Effect of fern extract on the PTP

Fern extracts contain factors that inactivate thiamine, one of which has been identified as thiaminase I (Evans 1975). Berlier and Bonnygri (1967) have claimed that caffeic acid is the active antithiamine principle.

The fern extract had an effect similar to that of pyridoxamine (Fig. 3) (Table I). The PTP was lost 20–40 min after injection of the extract. If the extract was heated to 100°C for 10 min, 4–7 times larger volumes of the extract had to be injected to get the same effect as that obtained with the unheated extract ($n = 3$). The active agent was found to be non-dialyzable.

TABLE I The effects of pyriithiamine, oxythiamine fern extract and d-tubocurarine on PTP are shown. The PTP is calculated by measuring the increase in height of the first 5 potentiated muscle twitches whereafter it is expressed as the ratio between the mean increase in the 5 muscle twitches and a supramaximal nonpotentiated single twitch. In the case of d-tubocurarine where total abolishment of the PTP always could be obtained by an additional dose if necessary the effect of the dose necessary for a total block of the PTP is compared with the effect on the single muscle twitches. A negative value on the PTP means that the post-tetanic twitches are smaller than the pretetanic twitches. The single muscle twitches (in the case of d-tubocurarine) are calculated as the ratio between the twitches after administration of curare and before.

Drug	Size of PTP before drug administration	Size of PTP after drug administration	Size of PTP after thiamine administration	Effect on single muscle twitches
Pyriithiamine	0.39	0.01	0.32	—
(50 mg/kg)	0.24	0.04	0.26	—
N=6	0.41	-0.04	0.28	—
	0.18	0.09	0.15	—
	0.37	-0.03	0.31	—
	0.26	0.04	0.32	—
Oxythiamine	0.58	0.58	—	—
(2 g/kg)	0.41	0.36	—	—
n=4	0.21	0.26	—	—
	0.47	0.39	—	—
Fern extract	0.45	-0.06	0.50	—
n=4	0.28	0.07	0.17	—
	0.58	0.05	0.36	—
	0.34	-0.01	0.15	—
d-Tubocurarine	0.36	0.00	—	0.98
(2-6 mg/kg)	0.32	0.01	—	0.92
n=4	0.45	-0.02	—	0.96
	0.22	0.00	—	0.95

I.v. injection of thiamine (10-20 mg/kg) restored the PTP (Fig. 3). Caffeic acid did not affect the PTP in doses at least up to 4 g/kg.

Effect of d-tubocurarine on the PTP

The effect of d-tubocurarine was investigated in order to compare its effect with the effects of pyriithiamine and fern extract.

a before



b after oxythiamine



Fig. 2. Effect of oxythiamine. PTP a) before injection of oxythiamine b) 6 min after the injection of oxythiamine (2 g/kg). No effect on PTP can be seen.

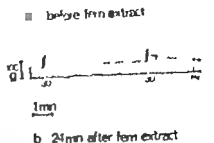
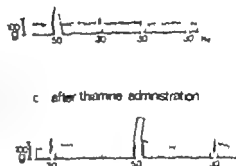


Fig. 3 Effect of fern extract on PTP. Single muscle twitches are evoked by stimulation at 30 Hz for 5 s) before injection of fern extract. The PTP as provoked by stimulating at 30 Hz for 5 s, b) 24 min after the injection of fern extract (7.5 ml). PTP has disappeared both after stimulation at 30 Hz for 20 sec and at 30 Hz for 5 s, c) 6 min after the administration of thiamine (10 mg/kg) PTP has reappeared. The PTP provoked by stimulation either at 30 Hz for 5 s or at 30 Hz for 20. The contraction force during the 30 Hz stimulation reached the maximum level for the time. Therefore the peak tension during these tetani cannot be calculated. In addition, the contractions during the 30 Hz tetani were not isometric since the initial tension was exceeded.



The PTP was inhibited to 50% by d-tubocurarine (2-6 $\mu\text{g}/\text{kg}$; range of 4 expts.) (Fig. 4). At these doses the ordinary twitches were unaffected. A dose of d-tubocurarine which caused 100% inhibition of the PTP reduced the ordinary twitches by ~8% only (range of 4 expts.) To inhibit the ordinary twitches by 50%, doses of 45-75 $\mu\text{g}/\text{kg}$ were required (range of 4 expts.). When the muscle twitches were abolished by a high dose of d-tubocurarine (5 mg/kg) PTP still occurred after direct muscle stimulation (Fig. 4) (n = 3).

Pyruvate determination

In thiamine deficiency pyruvate levels increase in the blood because of deficient pyruvate decarboxylation (Gubler 1967). The influence of thiamine antagonists on the coenzyme function of Th-DP was therefore followed by measuring the pyruvate levels in the blood.

Fig. 4 Effect of d-tubocurarine on PTP and muscle twitches after high frequency stimulation. Single muscle twitches were evoked by stimulating at 0.2 Hz. PTP as provoked by stimulating at 30 Hz for 5 s and the start-cross effect by continuous stimulation at 0.5, 1.0, 1.4, 4.0 and 8.0 Hz (and 16 Hz after copious administration). a) before injection with d-tubocurarine, b) after the injection of d-tubocurarine (3 $\mu\text{g}/\text{kg}$), c) direct stimulation of the sartorius muscle after the injection of 5 mg/kg d-tubocurarine. Notice that the magnification in (b) is higher than in (a) and (c).

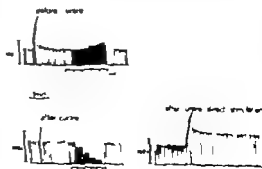


TABLE II Levels of thiamine and its phosphate derivatives ($\mu\text{mol/g}$ masseter muscle) before and 15, 30, 45, 60 and 75 min after the injection of thiamine antagonists. The mean, standard deviation and number of experiments are shown. The biopsies taken at different time intervals are from the same muscle. The controls show thiamine and thiamine-phosphate levels in muscles which are stimulated in the same manner as those which received the drugs.

	Before injection	15 min after injection	30 min after injection	45 min after injection	60 min after injection	75 min after injection
Pyrithiamide (50 mg/kg)						
Thiamine	0.29 ± 0.09 n=14	0.39 ± 0.14 n=9	0.24 ± 0.11 n=9	0.17 ± 0.08 n=11	0.11 ± 0.03 n=7	0.08 ± 0.02 n=8
Thiamine-phosphates	0.89 ± 0.16 n=14	0.80 ± 0.15 n=9	0.78 ± 0.14 n=9	0.71 ± 0.15 n=11	0.58 ± 0.37 n=7	0.55 ± 0.18 n=8
Oxythiamine (1 g/kg)						
Thiamine	0.29 ± 0.09 n=14		0.27 ± 0.10 n=5			0.24 ± 0.07 n=5
Thiamine-phosphates	0.89 ± 0.16 n=14		0.88 ± 0.14 n=5			0.81 ± 0.17 n=5
Controls, thiamine						
Thiamine	0.31 ± 0.14 n=5	0.28 ± 0.11 n=5	0.33 ± 0.16 n=4	0.28 ± 0.13 n=5	0.34 ± 0.16 n=5	0.34 ± 0.18 n=4
Thiamine-phosphates	0.96 ± 0.27 n=5	0.92 ± 0.22 n=5	0.87 ± 0.29 n=4	0.93 ± 0.31 n=5	0.85 ± 0.27 n=5	0.93 ± 0.21 n=4

The mean level of pyruvate in the blood before injecting thiamine antagonists was 169 ± 24 $\mu\text{mol/l}$ (mean \pm S.D. n=11). Pyrithiamine in doses which abolished the PTP (50 mg/kg) did not significantly change the level of pyruvate 174 ± 29 $\mu\text{mol/l}$ (mean \pm S.D., n=8) (measured 80 min after the injection of pyrithiamine). In contrast, the pyruvate level after oxythiamine treatment (2 g/kg) increased to 341 ± 67 $\mu\text{mol/l}$ (mean \pm S.D., n=3) (measured 60 min after the injection of oxythiamine).

Determination of thiamine and its phosphate ester

The levels of thiamine and its phosphate esters in the muscle biopsies before and after injection of thiamine antagonists are shown in Table II. Pyrithiamine decreased the levels of thiamine ($p < 0.01$) and of the thiamine phosphates ($p < 0.05$). Oxythiamine had no significant effect.

Discussion

When a skeletal muscle is stimulated at low frequency (0.1–0.2 Hz) with impulses of supra maximal voltage, maximal muscle twitches develop (Brown, Dale and Feldberg 1936). A change in the size of the muscle twitches can be used as an indicator of the efficiency of the neuromuscular transmission (Brown, Dale and Feldberg 1936). If the muscle is stimulated continuously at higher frequencies, then the muscle twitches in fast mammalian muscle will increase (stair-case effect) (Standaert 1964). If the low-frequency induced muscle twitches are interrupted by a short tetanic stimulation, then post-tetanic potentiation (PTP) of the single muscle twitches induced by low-frequency stimulation is seen (Brown and Euler 1938). The PTP is due to an increase in the contractile properties of the muscle fibres (Brown and Euler 1938, Standaert 1964).

The rat masseter muscle used in the present study is a typical "fast" muscle since it responds with individual twitches up to frequencies of 30 Hz. In agreement with the above

mentioned data it was found that the individual muscle twitches increased at high stimulation frequencies, and that PTP developed after moderate tetanic stimulations.

Administration of pyridoxamine or fern extract abolished the PTP after 40–80 min respectively. At the same time a frequency-dependent depression of the muscle twitches was seen (Fig. 1). Thus pyridoxamine caused the fast muscle to behave as a slow muscle. A very similar change was produced by d-tubocurarine (Fig. 4). It has been shown (Bochthal and Lindhard 1937, Engbeck 1948, Naeve 1952, Lillichal and Naeve 1961, Bowman and Webb 1973) that the frequency-dependent depression of the neuromuscular transmission is due to a presynaptic effect of d-tubocurarine: the margin of safety for the conduction of trains of nerve impulses in the terminal motor nerve arborization is reduced. Hubbard and Schmidt (1961) and Hubbard *et al.* (1965) showed that d-tubocurarine reduced the magnitude and duration of the negative after-potential in the nerve endings of the phrenic nerve-diaphragm preparation, effects antagonized by neostigmin. These results suggest that the negative after-potential and the presynaptic spike are influenced by acetylcholine. Beranek and Vysokil (1967) and Hubbard *et al.* (1969) showed that the presynaptic effects by d-tubocurarine were frequency-dependent: the end plate potential was depressed during repetitive stimulation by d-tubocurarine in concentrations that did not abolish depolarization produced by an iontophoretic application of acetylcholine. In spite of the similarities between the effect of pyridoxamine and d-tubocurarine it is improbable that the effects of pyridoxamine described are due to a direct curare-like action of pyridoxamine or the fern extract since curare-like drugs act within a few minutes (Paton and Zaima 1951). The observation that the effects of pyridoxamine and fern extract were reversed by thiamine are in accordance with the assumption that thiamine is essential for the muscle twitches in the preparation studied. Pyridoxamine did not affect the muscle twitches elicited by direct muscle stimulation. Therefore it is probable that thiamine is important for the neuromuscular transmission.

In an earlier study (Waldenkind *et al.* 1978) it was shown that thiamine in high doses has depressive action on the miniature end plate potentials. Since such an action cannot account for the effects in the present study a presynaptic effect seems as the most probable explanation for the thiamine effect. However the specific binding of thiamine to nicotinic receptors (Waldenkind *et al.* 1978) could be in agreement with the view that thiamine binds to "presynaptic alkaline receptors" and is of importance for their function (nicotinic receptor is defined as a structure which binds d-tubocurarine in low concentrations).

Pyridoxamine has been shown to abolish post-tetanic hyperpolarization (the positive after potential) in unmyelinated C-fibres of rabbit vagus nerve (Armstrong and Cooper 1965). Such a change in membrane potential could be due to an impairment of the active transport of sodium and potassium that regenerates and maintains the membrane potential. Barchi (1976) showed that thiamine preserves the electric field across excitable membranes of the node of Ranvier a process which was strongly temperature dependent thereby suggesting an involvement of a chemical reaction. He also noticed an increase in the refractory period, a widening of the action potentials and depolarizing after-potentials after pyridoxamine treatment, results which could be in accordance with the view that the active transport of sodium and potassium by the Na^+K^+ ATPase across the membrane was disturbed. An impairment of the active transport of ions will lead to difficulties in maintaining the membrane potential

TABLE II Levels of thiamine and its phosphate derivatives (nmol/g masseter muscle) before and 15, 30, 45, 60 and 75 min after the injection of thiamine antagonists. The mean, standard deviation and number of experiments are shown. The biopsies taken at different time intervals are from the same muscle. The controls show thiamine and thiamine-phosphate levels in muscles which were stimulated in the same manner as those which received the drugs.

	Before injection	15 min after injection	30 min after injection	45 min after injection	60 min after injection	75 min after injection
Pyriethiamine (50 mg/kg)						
Thiamine	0.29 ± 0.09 n=14	0.39 ± 0.14 n=9	0.24 ± 0.11 n=9	0.17 ± 0.08 n=11	0.11 ± 0.03 n=7	0.08 ± 0.02 n=8
Thiamine- phosphates	0.89 ± 0.16 n=14	0.80 ± 0.15 n=9	0.78 ± 0.14 n=9	0.71 ± 0.15 n=11	0.58 ± 0.37 n=7	0.55 ± 0.18 n=8
Oxythiamine (1 g/kg)						
Thiamine	0.29 ± 0.09 n=14		0.27 ± 0.10 n=5			0.24 ± 0.07 n=5
Thiamine- phosphates	0.89 ± 0.16 n=14		0.88 ± 0.14 n=5			0.81 ± 0.17 n=5
Controls, thiamine						
Thiamine	0.31 ± 0.14 n=5	0.28 ± 0.11 n=5	0.33 ± 0.16 n=4	0.28 ± 0.13 n=5	0.34 ± 0.16 n=5	0.34 ± 0.16 n=4
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The mean level of pyruvate in the blood before injecting thiamine antagonists was 169 ± 74 μ mol/l (mean \pm S.D. n=11). Pyriethiamine in doses which abolished the PTP (50 mg/kg) did not significantly change the level of pyruvate 174 ± 29 μ mol/l (mean \pm S.D. n=8) (measured 80 min after the injection of pyriethiamine). In contrast the pyruvate level after oxythiamine treatment (2 g/kg) increased to 341 ± 67 μ mol/l (mean \pm S.D., n=3) (measured 60 min after the injection of oxythiamine).

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References

- ARMETT CH J and J M RITCHIE, The action of acetylcholine on conduction in anastomosis non-myelinated fibres and its prevention by an acetylcholine-esterase. *J Physiol (Lond)* 1960, 112, 141-158.
- ARMETT CH J and J M RITCHIE, The action of acetylcholine and some related substances on conduction in the smaller non-myelinated nerve fibres. *J Physiol (Lond)* 1961 133 372-384.
- ARMETT CH J and J R COOPER, The role of thiamine in nervous tissue: effect of antineurotoxicity of the vitamin on conduction in anastomosis non-myelinated nerve fibres. *J Pharmacol. exp Ther* 1965, 149 137-143.
- BANCROFT, R L, The somatotropic role of thiamine in excitable membrane function. In *Thiamine* Ed. by C J Gablet M Figgars and P M Dreyfus John Wiley & Sons, New York 1976 233-305.
- BRANKE, R and F VYSOCKI, The action of tubocurarine and atropine on the normal and desensitized rat diaphragm. *J Physiol (Lond)* 1967 123 53-66.
- BRUCE, J and J C BANCROFT, 3,4-Dihydroxybenzoic acid, an antithiamine factor of fern. *Experientia (Basel)* 1967 23, 996-997.
- BROWN, W C and S H WILK, Acetylcholine and anticholinesterase drugs. In *Neuromuscular Blockade & Stimulating Agents* Ed Chrysiol Pergamon Press, 1973 II, 473-902.
- BROWN, G L, H K DALL and W FULMER, Reactions of the cortical cholinergic system to acetylcholine and to curare. *J Physiol (Lond)* 1936, 87 394-424.
- BROWN, G L and U S VON EDLER, The after effects of a vitamin on mammalian muscle. *J Physiol (Lond)* 1932 85 39-60.
- BUCHTHAL, F and J LINDHARD, Direct application of acetylcholine to motor end plates of skeletal muscle fibres. *J Physiol (Lond)* 1957 90 32-43P.
- ECCLER, J C *The Physiology of S. aureus* Springer Verlag 1964 P 99.
- ENGLEKE, L, Investigations on the course and localization of magistralis esterase. A comparison with other enzymes. *Acta pharmaceut. (Kbh)* 1942, 1 Suppl. 21.
- EWING, W C *Thiamine and their effects on animals. Vitamins and Hormones*, 1975 447-504.
- GERARD, P and H W STRAUB, After-potentials in mammalian non-myelinated nerve fibres. *J Physiol (Lond)* 1958 144 442-462.
- GUTLER, C L *Ecstasy studies in thiamine deficiency I* *Thiamine* Ed by C J Gablet M Figgars and P M Dreyfus, John Wiley & Sons, New York, 1976 121-139.
- HORTHUM, B T ERICK and W MILES, Über Katalase des Thiamin- und tri-phosphorantagonist mit Niacin- Kation, und Calcium ionen. *Festschr-Schweiz. Z. physiol. Chem.* 1964 323, 156-159.
- HOWARD, J L and R F SCHWEDT *Stimulation of motor nerve terminals. Nature (Lond)* 1961 191 1003-1004.
- HOWARD, J L R F SCHWEDT and T YOSOTA, The effect of acetylcholine upon mammalian motor nerve terminals. *J Physiol (Lond)* 1965 181 810-820.
- HUBBARD, J I D P WILSON and M MIYAMOTO, Reduction of transmitter release by d-tubocurarine. *Nature (Lond)* 1969 221 931-933.
- KODIAM, J C *The mode of action of pyridoxine as an indicator of thiamine deficiency. Biochem. Aspects. Acta (Amst)* 1958 29 333-344.
- LELIEUX, G and K NAEM, A presynaptic effect of d-tubocurarine in the neuromuscular junction. *Acta physiol. scand* 1961 32 23-31.
- MURAL, A, von, Thiamine and peripheral neurophysiology. *Vitamins and Hormones* 1947 93-118.
- NAEM, K, The mechanism of action of curare. *Acta pharmaceut. (Kbh)* 1972, 2, 149-163.
- P TON, W D and E J ZAROS, The action of d-tubocurarine and of decamethonium on respiratory and other muscles in the cat. *J Physiol (Lond)* 1951 112, 311-331.
- RITCHIE, J M and K W STRAUB, The hyperpolarization which follows activation in mammalian non-myelinated fibres. *J Physiol (Lond)* 1957 106 80-97.
- SEAL, S A E BLACK and S B WYNDALE, An electrolytic spectrophotometric method for the determination of pyruvic acid in blood. *J Lab. clin. Med.* 1936, 48 157-163.
- ST. LAURENT, F O *The mechanisms of post-tetanic potentiation in cat soleus and gastrocnemius muscles. J gen. Physiol* 1964, 47 967-1001.
- STRAUB, E, The mode of action of some thiamine analogues with antithiamine activity. In *Thiamine Deficiency* Ciba Foundation Study Group No. 28 Ed by G. E. W. Wolstenholme and M. O'Connor Churchill Ltd, London 1967 26-42.

and thereby the neurotransmission especially at higher stimulation frequencies. Therefore the present results can be in agreement with the view that thiamine is of importance for the active transport of ions through the nerve terminal membrane.

Since the effects of pyridithiamine and d-tubocurarine on PTP in rat masseter muscle were similar it would be of interest if acetylcholine, in addition to its effects on the muscle end plate, has an action on the positive after potential (and thereby on the active transport of sodium and/or potassium) in analogy with pyridithiamine. Acetylcholine has in fact been shown to increase the post-tetanic hyperpolarization (positive after potential) in mammalian nonmyelinated nerve fibres (Armstrong and Ritchie 1960). The effect of acetylcholine was inhibited by d-tubocurarine (Armstrong and Ritchie 1960). The positive after-potential is caused by the same mechanism as the post tetanic hyperpolarization which is due to an active transport of potassium into the axons (Greengard and Straub 1958). It is therefore possible that d-tubocurarine might affect the degree of polarization of the nerve membrane by interfering with the active transport of sodium and potassium through the nerve membrane a transport which in some way could be under the influence of acetylcholine at high frequency stimulation.

Both d-tubocurarine and pyridithiamine inhibit the post-tetanic hyperpolarization of the nerve membrane and might therefore decrease transmitter output during high frequency stimulation. The transmitter output is directly dependent on the degree of polarization of the nerve membrane (Eccles 1964). The assumption that the abolishment of the PTP is due to a block of a presynaptic function of acetylcholine which is related to membrane polarization (Armstrong and Ritchie 1960, 1961) is in accordance with the results where it was shown that PTP was blocked by d-tubocurarine, hexamethonium and AChE-inhibitors (Waldenlind 1978). In other words the PTP and the membrane polarity after nerve stimulation are sensitive to the same types of drugs.

The mechanism by which thiamine would maintain and regenerate the membrane potential remains unknown. Thiamine could be of importance for acetylcholine synthesis, for generation of ATP to the ion "pump" it could act as an ion carrier as was suggested by Hoffman *et al.* (1964) or it could be of importance for the function of the "presynaptic nicotinic receptors" (Waldenlind 1977). Pyridithiamine is a potent inhibitor of thiamine kinase—an enzyme converting thiamine to Th-DP (Steyn Parvé 1967). Th-DP is, according to Koedam (1958), the form in which thiamine is stored in the body. He therefore suggested that pyridithiamine interferes with the phosphorylation of thiamine and thereby with the storage of the vitamin in the body. In the present study a decrease of both thiamine and Th-DP was noted in the masseter muscle (Table I). Pyridithiamine administration did not elevate the pyruvate levels in the blood but abolished the PTP. Oxithiamine administration, however, increased the pyruvate levels of the blood but had no effect on the PTP. If the blood levels of pyruvate should reflect the metabolism in the nerve terminals, these results would suggest that the action of thiamine is unrelated to the coenzyme functions of Th-DP. However, it is uncertain that a lack of effect on the levels of pyruvate in the blood shows that pyruvate dehydrogenase in the nerve terminals is unaffected. Therefore an impairment of the metabolic reactions requiring Th-DP may still be the ultimate mechanism behind the observed effect of pyridithiamine and fern extract on neurotransmission.

Correlation between fluid reabsorption and proximal tubule ultrastructure during development of the rat kidney

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ANITA APERIA and LARS LARSSON

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Abstract

APERIA, A. and L. LARSSON. Correlation between fluid reabsorption and proximal tubule ultrastructure during development of the rat kidney. *Acta physiol. scand.* 1979 105: 11-22.

Parallel functional and ultrastructural studies were performed in maturing rats in order to locate factors determining the development of proximal tubular fluid reabsorption. Three groups of hydropenic animals, born on 22 to 24, 28 to 33 and 40 to 43 days old, are studied. Nephron function was estimated at the single nephron level by micropuncture technique. The ultrastructure of the developing proximal tubules was analysed by morphometric techniques following fixation of single nephrons. Kidney eight proximal convoluted tubule length and diameter increased during postnatal development. SNGFR increased from 2.36 to 3.57 and to 20.5 nl/min in respective groups of rats. Proximal tubular fluid reabsorption J_v (μ) increased from 0.15 to 0.22 and 0.38 $\mu\text{m}^2 \cdot \mu\text{m}^{-1} \cdot \text{s}^{-1}$. Parallel to the functional development the relative area of lateral and basal cell membrane increased, resulting in constant relationship between net fluid reabsorption and the lateral and basal cell membrane area during the fourth postnatal week and then only slight increase in this relation during the further development. The results suggest that net fluid transport during hydropenia is determined by the amount of available lateral and basal cell membrane where the transporting enzyme for sodium is located.

Key words: Cell membrane and fluid reabsorption, renal structural and functional development, micropuncture of developing tubules, single nephron glomerular filtration during development, Sprague Dawley rats

Clinical studies on the development of renal function have shown that kidneys in preterm infants have a reduced capacity to retain sodium during negative sodium balance as well as to excrete sodium during positive sodium balance (Aperia *et al.* 1972, Aperia *et al.* 1975). Since this homeostatic insufficiency cannot only be explained by a low glomerular filtration rate (Aperia *et al.* 1975), sodium and thereby fluid reabsorption in the immature kidney must during various physiological conditions differ in one or several aspects from that of the mature kidney.

Developmental (Horster and Valtin 1970, Spitzer and Brandts 1974) as well as structural (Heber 1905, Larsson 1975) studies on renal tubules are complicated by the fact that at given age different nephron generations attain different maturational levels. In addition, the characteristic pathway for different transport systems vary in different parts of the nephron. For these reasons it is necessary to restrict each study of mechanisms

- WALDENLIND L., Release of thiamine and formation of a methylthiamine-like substance in the phrenic nerve diaphragm preparation of the rat *Acta physiol. scand.* 1977. *101* 22-27.
- WALDENLIND L., An Improved method for the determination of endogenous thiamine and its phosphate esters in biological material. *Nutrition and Metabolism*. In press.
- WALDENLIND L., L. ERFMAN and B. RYDQVIST. Binding of thiamine of the isolated nicotinic receptor and effects of thiamine on miniature end plate potentials of the frog. *Acta physiol. scand.* 1978. *103* 154-159.
- WALDENLIND L., Pharmacological properties of nicotinic receptors mediating post-tetanic potentiation in rat masseter muscle *Acta physiol. scand.* 1978. *103* 160-164.

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- WALDENLIND, L., An improved method for the determination of endogenous thiamine and its phosphates in biological material. *Nutrition and Metabolism*. In press.
- WALDENLIND, L., L. ELFMAN and B. RYDQVIST. Binding of thiamine of the isolated nicotinic receptor and effects of thiamine on miniature end plate potentials of the frog. *Acta physiol. scand.* 1978. 103: 154-159.
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radon (λ) the fluid reabsorption per unit luminal surface area as calculated (Gydy 1971) as

$$J_v(\lambda) = \frac{0.347}{T/12} (\mu\text{m}^3 \mu\text{m}^{-2} \text{sec}^{-1})$$

Estimations of the diameter in the oil perfused tubule will be referred to as stop flow determinations.

The reabsorptive rate of the proximal tubule was recorded in eight 22- to 4-day-old rats (group I), in eight 28- to 31-day-old rats (group II) and in five 40- to 45-day-old rats (group III). In the majority of the animals, determinations of total glomerular filtration rate, GFR, and SNGFR, were also made. In 4 animals in group I, in three animals in group II and in two animals in group III, the functional study as followed by fraction of proximal tubule. In addition, proximal tubules are prepared for electron microscopy studies in one III to 24-day-old rat, three 28- to 31-day-old rats and two 40- to 45-day-old rat which had not been subjected to previous micropuncture studies. The preparations, including catheterizations, infusion and dissection of the left kidney were, however identical in all rats studied.

Ultrastructural studies The tubules were fixed *in situ* by micropuncture of single proximal tubule (Marschack 1966) with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 0.5% lanthanum green at pH 7.2. The tubules were located while the same region as those used in the functional experiments and they were perfused with the fixative during 2 min. A small piece of the kidney containing the fixed tubule was excised and fixed for an additional 60 min in the same fixative used for perfusion, post fixed in osmium tetroxide in Veronal acetate buffer (pH 7.2) before dehydration in acetone and embedding in Vestopal W. Ultrathin sections are cut with LKB Ultratome III, stained with lead citrate and analyzed in JEOL JEM 100 B or Philips 300 electron microscope.

Electron micrographs at final magnification of 13 000 were mounted together so that each montage usually covered whole proximal tubule cross-section. Montages from at least 4 tubules in each age group (see above) are analyzed by morphometric analysis (Weibel *et al.* 1966). A square test lattice was superimposed on the montages and intersections between test lines and lateral and basal cell membranes are counted. The distance between the lines of the lattice corresponded to 0.6 μm on the electron micrograph. The relative area of lateral and basal cell membranes as μm^2 per μm^2 cell volume (surface density) is calculated from the formula given by Weibel *et al.* 1966, $S_v = 2N/L_T$, here N the number of intersections between the contour of the surface (cell membrane) and test lines, L_T the total length of the test lines expressed in μm . The relative volume of mitochondria as determined by point counting (Weibel *et al.* 1966). The same test lattice was utilized for this purpose and the intersections between the testlines were used as points. The significance of the difference between the means was evaluated by the Student's *t*-test.

Diameter of proximal tubule lumen The luminal diameter of the proximal tubule during free flow was determined in three 22- to 24-day-old rats, in three 28- to 31-day-old rats and in three 40- to 45-day-old rats. For this purpose the rats were prepared for micropuncture studies as described above. Photographic recording at 80 times magnification were made 10-15 seconds following the insertion of a 10 μm lanthanum green solution (10 μl /100 μg rat) into femoral artery catheter that had been advanced into the aorta. In some, the extremities of the renal artery. The diameter of randomly selected tubules as measured at 400 times magnification. The diameter of the proximal tubule lumen during stop flow as measured as the width of the oil drop in the light micrographs taken on the tubules whose reabsorption capacity was determined.

Length of the proximal tubule Dissection of proximal tubules as carried out in three rats in each age group. For this purpose the left kidney was removed and digested in 20% hydrochloric acid for 5 h at 37°C. The digested kidney was placed in distilled water. In each kidney four superficial nephrons were dissected and placed intact with the glomerulus and the entire proximal tubule in a drop of distilled water. A camera lucida drawing at 80 times magnification was made of the entire convoluted tubule. The length of the tubule was determined by tracing the drawing with thread.

Results

The postnatal development of some growth characteristics of the rat kidney during the period between 22-45 days are shown in Table I. The weight of the kidney increased 2.7-fold from 0.34 g to 0.92 g. The length of the proximal tubule (1st and 2nd segment excluding the 3rd segment, pars recta) increased 1.5-fold, as judged from micro-dissected tubules. The growth in length follows approximately the same pattern as that previously reported in

governing the development of renal sodium absorption to a specific segment of one nephron generation

This report describes the development of the intrinsic reabsorptive capacity of the second segment of the rat proximal tubule (Maunsbach 1966 b) and relates the functional development to the ultrastructural maturation of the proximal tubular cells.

The information obtained will enable a better understanding of the mechanisms governing fluid reabsorption during hydropenia in the maturing rat.

Materials and methods

Experiments were performed in 22 to 45-day-old male Sprague Dawley rats. The rats weighed between 50 and 220 g. They received an ordinary laboratory diet and fluid *ad libitum*. Four hours prior to starting the experiments food and fluid were removed from the cage. The animals were anesthetized with Inactin (Promonta, West Germany) intraperitoneally (80 mg/kg). They were then intubated and placed on a thermostatically controlled micropuncture table. One carotid artery and one jugular vein were cannulated with fine polyethylene catheters. The venous catheter was used for continuous infusion of loxalen, Inactin (Laevosan Gesellschaft Linz, Austria) 25 μ l normal saline which was given at a rate of 10 μ l/g rat/hour. The continuous infusion was preceded by a priming dose of the same solution (10 μ l/g rat). The arterial catheter was used for blood sampling and pressure recording. Arterial blood pressure was checked regularly and only those preparations were used in which the pressure was maintained throughout the experimental procedure. A stream of 100% oxygen continuously passed in front of the intubation tube. In this way the arterial oxygen tension of the animals was maintained between 85 and 100 mmHg.

The animals were prepared for micropuncture study using the left flank approach. Detailed information of the micropuncture procedure used in this laboratory has been given previously (Apéria and Henn 1975). The left kidney was placed in a Lucat cup. A fine polyethylene catheter was inserted into the lower 5–10 mm below the pelvis. The outer diameter of the catheter ranged between 0.46 and 0.48 mm, whereas the inner diameter of the catheter ranged between 0.40 and 0.42 mm. Single nephron glomerular filtration rate (SNGFR) was determined by spontaneous fluid collection from three to six randomly chosen proximal tubules in each rat. It was determined in tubular fluid samples spectrophotometrically using a micromodification of the a thron method (Hügel et al. 1958). Details of the method used have been described previously (Apéria and Henn 1975). At the time of tubular fluid collection for SNGFR determination, one or two blood samples (20–30 μ l each) were taken from the arterial catheter for determination of inulin concentration.

The reabsorptive rat. The proximal tubule was determined with a modification of the Gertz technique (Gertz 1963). In two to six tubules in each rat early parts of the second segment of the proximal tubule, which had a straight appearance, were punctured with a single bevelled sharpened glass capillary with a tip diameter of 6–7 μ m. The glass capillaries were filled with Sudan stained mineral oil. The approximate location of the puncture site was determined by letting a small oil drop pass through the tubule. An oil drop with the length of at least 4 tubular diameters was then injected into the lumen and was allowed to pass 2.5–4 tubular diameters beyond the capillary tip. Thereafter another oil drop of at least 3 tubular diameters in length was injected into the tubular lumen. The fluid between the two oil drops thus consisted of primary urine. The oil drops surrounding the drop of tubular fluid were kept in place or flowed to advance slowly by changing the pressure in the puncturing pipette. When the drops were in the desired position, the dimming length of the urine drop surrounded by the oil drops was followed by the time sequence photomicrography at 4 sec intervals. For this purpose Zeiss Contarex motor driven camera applied to a Zeiss stereomicroscope was used. The lens system permits a magnification of 80 times. Illumination was provided by a 60 watt/h flash unit synchronized with the camera. The negatives of each exposure were magnified 5 to 7 times. The lengths of the epithelial outlines of each urine drop, the distance between the bases of the opposing oil menisci was then measured and the mean value for the 2 outlines lengths (L) was used. The measurement of L was plotted semilogarithmically against time and the reabsorptive half-time ($T_{1/2}$) of the droplet could then be determined. The following criteria were used for accepting recording: 1) The tubular diameter was constant throughout the procedure indicating a constant intratubular pressure. 2) The initial length of the urine droplet was more than 50 μ m. Estimations of linearity were generally confined to the intermediate phase of shrinking. From $T_{1/2}$ and the tubular

TABLE III Filtration rate and proximal tubule fluid reabsorption in all rats subjected to micropuncture studies.

Age days	SNQFR nl/min	QFR/ μ KW nl/min g	J_v () μ ml/ μ m ² /sec ⁻¹
Group I			
23	(—)	(—)	0.11
23	1.92	0.39	0.13
23	(—)	(—)	0.13
23	2.59	0.53	0.16
23	2.96	0.57	0.17
23	3.66	0.36	0.16
24	3.74	0.60	0.19
24	2.96	0.49	0.18
Mean value \pm S.D.	2.98 \pm 0.66	0.49 \pm 0.10	0.15 \pm 0.03
Group II			
29	3.90	0.66	0.23
29	(—)	(—)	0.17
29	(—)	(—)	0.16
30	6.72	0.71	0.24
30	11.00	0.79	0.20
30	7.00	0.79	0.25
31	11.80	0.84	0.23
31	9.19	0.80	0.21
32	(—)	(—)	0.27
Mean value \pm S.D.	8.37 \pm 2.46	0.76 \pm 0.07	0.22 \pm 0.04
Group III			
40	16.3	0.79	0.32
41	19.7	0.99	0.32
41	22.1	1.62	0.32
42	22.0	0.96	0.32
42	22.2	1.30	0.39
Mean value \pm S.D.	20.3 \pm 2.6	1.01 \pm 0.19	0.34 \pm 0.03

The general ultrastructure of tubules prepared for electron microscopy appeared well preserved as shown in Fig. 2. The tubules examined with the electron microscope were almost circular in cross section and were surrounded by a complete basement membrane of about 0.6 μ m in thickness in the most mature tubules. The cells at all developmental stages were about 8 μ m in height and exhibited a brush border of constant length. The microvilli of the brush border were about 1.2 μ m in the 22- to 24-day-old rats and about 1.5 μ m in older rats. The increase in the height of the microvilli and the increase in tubular diameter during the development of the proximal tubule resulted in an absolute increase of the apical cell membrane area between groups I and II with a factor 1.5 and between groups II and III with a factor 1.1 per mm tubule.

Mitochondria in the most mature tubules were oriented perpendicular to the basement membrane and in close relation to lateral and basal cell membranes. In the younger tubules, such an obvious orientation of the mitochondria was not present although some of them tended to be oriented in a similar way as in the adult (compare Fig. 3 a and b). The relative volume of the mitochondria (Fig. 4 a) did not change from 23 to 30 days of age. From 30 to 42 days of age the relative mitochondrial volume increased 1.3-fold whereas the absolute volume of the mitochondria increased linearly during the entire development (Fig. 4 b).

TABLE I Size of the kidney and the proximal tubule in 22 to 45-day-old rats. Values are mean \pm S.D.

Age (days)	Group I 22-24	Group II 28-32	Group III 40-45
Kidney weight, g	0.34 \pm 0.04 (8) ^a	0.53 \pm 0.06 (9) ^a	0.92 \pm 0.06 (5) ^a
Length of convoluted proximal tubule mm	4.83 \pm 0.32 (12) ^b	5.59 \pm 0.55 (12) ^b	7.39 \pm 0.66 (12) ^b
Diameter of proximal tubule lumen during free flow μ m	12.8 \pm 1.2 (10) ^b	15.0 \pm 1.4 (20) ^b	16.4 \pm 1.9 (20) ^b
Diameter of proximal tubule lumen during stop flow μ m	25.1 \pm 1.1 (8) ^c	27.0 \pm 0.7 (9) ^c	29.5 \pm 0.9 (5) ^c

^a Number of rats. Only animals in which functional determinations were made, are included.

^b Number of tubules measured.

^c Number of rats. Only animals in which functional determinations were made are included. In each rat the mean value of 2-6 observations has been used.

Wistar rats (Wahl and Schnermann 1969). The measurement of the luminal diameter of the tubule showed larger values when measured between the oil drops, i.e. during stop flow condition than during free flow condition. This discrepancy has been observed previously (Lewy and Windhager 1968; Nakajima *et al.* 1970). During stop flow the oil drop will both flatten the brush-border (Langer *et al.* 1968) and distend the tubule which results in an increased diameter of the tubule lumen.

The fractional water excretion (V/GFR) 100% was used as an index of the state of hydration (Table II). (V/GFR) 100% was similar in each group and not significantly different from the finding in 40- to 45-day-old rats that had been fluid deprived for a longer time. It was considerably lower than in 40- to 45-day-old rats that had been fluid expanded with isotonic saline amounting to 3% of the BW.

Postnatal development of functional variables is shown in Table III. From 23 to 40 days of age, the amount of fluid reabsorbed per μ m of apparent proximal tubular length per second, J_v (s) increased 2.2 fold as also indicated by the fall in the half time for proximal tubule fluid reabsorption (Fig. 1). During the same age interval the filtered load in the superficial nephrons (SNGFR) increased 6.9-fold. Total GFR increased from 0.49 to 1.01 ml/g kidney weight/min. The development of total GFR in relation to kidney weight in the present study continues with approximately the same slope from that reported by Horster and Lewy (1970) from birth to 18-day-old rats. Total GFR per gram kidney weight thus increases rapidly up to about 30 days of age.

TABLE II. Fractional water excretion (V/GFR) 100%. Values are mean \pm S.D. Number of animals studied in parentheses.

Group I	0.65 \pm 0.18 (5)
Group II	0.79 \pm 0.18 (5)
Group III	0.61 \pm 0.27 (5)
40-day-old rats following	0.64 \pm 0.17 (5)
40-day-old rats following	7.14 \pm 1.64 (5)
390 min isotonic volume expansion	

TABLE III. Filtration rate and proximal tubule fluid reabsorption in all rats subjected to micropuncture studies.

	Age days	ENOFER ml/min	GFR/g KW ml/min g	Jv (x) mm ² /mm ² /sec
Group I	22	—	—	0.11
	22	1.92	0.39	0.13
	22	—	—	0.13
	23	2.99	0.53	0.16
	23	2.98	0.57	0.17
	23	3.66	0.36	0.16
	24	3.74	0.60	0.19
	24	2.96	0.49	0.18
Mean value ± S.D.		2.98 ± 0.48	0.49 ± 0.10	0.15 ± 0.03
Group II	28	5.80	0.66	0.23
	28	—	—	0.17
	29	—	—	0.16
	30	6.73	0.71	0.24
	30	11.00	0.79	0.20
	30	7.00	0.79	0.25
	31	11.80	0.84	0.23
	31	9.10	0.80	0.21
	32	—	—	0.27
Mean value ± S.D.		8.57 ± 2.46	0.76 ± 0.07	0.22 ± 0.04
Group III	40	16.3	0.75	0.33
	41	19.7	0.99	0.32
	41	22.1	1.02	0.32
	43	23.0	0.96	0.32
	43	22.2	1.30	0.39
Mean value ± S.D.		20.5 ± 2.6	1.01 ± 0.19	0.34 ± 0.03

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50 Reabsorptive halftime sec



Fig. 1 Proximal tubular reabsorptive halftime in seconds during postnatal development of 22 to 45-day-old rats.

The cells of the proximal tubules interdigitated more in mature tubules than in the immature tubules resulting in an increase in the area of the lateral and basal cell membranes per μm^3 cell volume (Fig. 5 a). The total volume of one mm tubule wall including the microvilli, i.e. the total volume of the cells in one mm tubule, was calculated from outer and inner tubular diameters determined at free flow conditions. (This procedure gives a 10% percentage overestimate of the cell volume since there are large interspaces between the microvilli of the brush border. Since this error is the same at all developmental stages comparison between data from the different developmental stages are still valid.) By multiplying the arrived value on total cell volume with the determined relative areas surface densities of lateral and basal cell membranes (Fig. 5) the total area of lateral and basal cell membranes per mm tubule was obtained in each developmental stage. This showed an increase in the total area of lateral and basal cell membranes by a factor 1.8 from the youngest to the most mature tubules (Fig. 5 b).

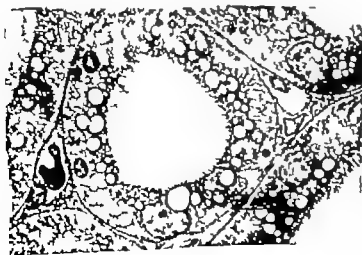


Fig. 2. Ultrastructural appearance of a proximal tubule from a 22-day-old rat perfused by microperfusion with glutaraldehyde. Note the even appearance of the microvilli of the brush border and the well preserved general ultrastructure of the cells. $\times 2000$.

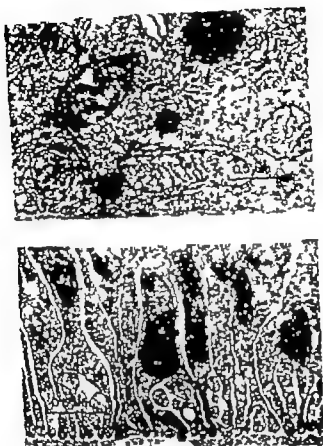


Fig. 3a. Basal part of proximal tubule cell from 22-day-old rat with relatively few mitochondria which have no obvious cristae. Note the few infoldings of the cell membrane (CM). Fig. 3b shows an area corresponding to that seen in Fig. 3a from 45-day-old rat with many mitochondria (M) which are perpendicularly oriented to the basement membrane (BM). Note the pronounced lateral infoldings of the cell membrane (CM). Both Figs. 3a and 3b are of the same magnification, 30 000.

Fluid reabsorption per unit apical area (excluding the area of the microvilli), $J_v(a)$, increased 2.2-fold. The apparent luminal area (excluding the area of the microvilli) in one mm tubule was obtained from the determination of the inner tubular diameter during free flow conditions. The relation between this area and the total area of the lateral and basal cell membranes in one mm tubule (calculated as above) was determined and used when calculating $J_v(B+L)$ from $J_v(a)$. $J_v(B+L)$ increased 1.5-fold from 23- to 42-day-old rats (Fig. 6). It should be noted that the most pronounced increase in $J_v(B+L)$ will occur between 30 and 42 days of age (1.5-fold, $p < 0.01$) while $J_v(B+L)$ does not change significantly from 23 to 30 days of age.

The individual relationship between water flux and basolateral surface membrane in those animals subjected both to functional and structural studies is shown in Fig. 7. The correlation coefficient $r = 0.83$ is significant.

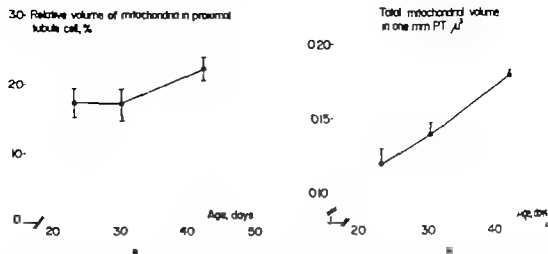
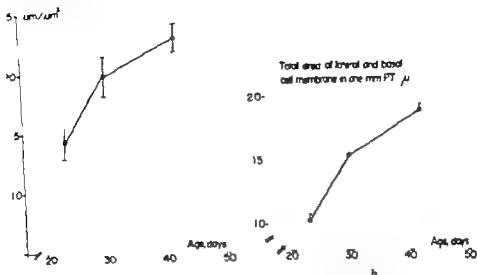


Fig. 4 a and 4 b Changes of mitochondrial volumes during the postnatal development of the proximal tubule. Fig. 4 a shows the relative mitochondrial volume and Fig. 4 b shows the total mitochondrial volume in one mm proximal tubule. Vertical bars indicate the standard error. The difference between groups II and III in Fig. 4 a is statistically significant ($p < 0.005$).

Discussion

The present study demonstrates a constant relationship between an increasing fluid reabsorption and area of lateral and basal cell membranes during the fourth postnatal week and then only a small increase in this relation during the further studied postnatal development of superficial proximal tubules. It is unlikely that the significant correlation between water flux and basolateral membrane surface is a chance occurrence, since a similar observation has been observed in the developing rabbit proximal tubule where fluid reabsorption has been determined *in vitro* where the tubules were isolated and perfused (Larsson and Horster 1976). It can therefore be suggested that fluid reabsorption in the developing proximal tubules at least during the studied condition of hydropenia may be dependent on the amount of available lateral and basal cell membranes. Most investigators seem to agree that fluid transport in adult proximal tubules will occur both across the epithelial cells (cellular pathway) and the intercellular junctions (paracellular pathway) and will be determined by the osmotic and hydrostatic pressure gradients between the tubular lumen and the peritubular spaces, including the intercellular spaces (Windhager and Giebisch 1976). Water will primarily be reabsorbed because of the osmotic gradient between the lumen and the intercellular spaces (Diamond 1969) created mainly by active transport of sodium to the intercellular spaces (Bank 1976, Green and Giebisch 1975), at least during hydropenia. The sodium transport is considered to be mediated by $(\text{Na}^+ + \text{K}^+) \text{-ATPase}$ (Skou 1965) which is located in the cell membrane (Kyte 1976, Sackin and Boulpaep 1975). The present observation of the parallel increase of fluid reabsorption and amount of lateral and basal cell membranes therefore indicates that the amount of sodium transporting enzyme is important for the increased fluid reabsorption. Since $(\text{Na}^+ - \text{K}^+) \text{-ATPase}$ was not determined biochemically, unequivocal evidence for this hypothesis is not yet available for developing rats. However, in keeping with this suggestion is result from recent studies in maturing rabbit proximal tubules analysed after isolation and perfusion *in vitro* (Horster

Area of lateral and basal cell membranes
per unit cell volume



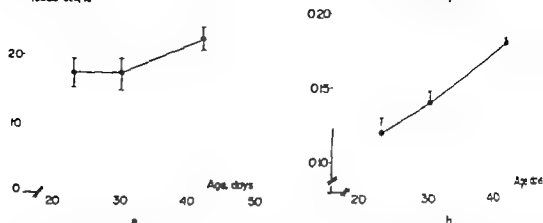
Figs. 5 and 5b. Area of lateral and basal cell membranes of the proximal tubule wall during postnatal development. Fig. 5 shows the area of lateral and basal cell membranes related to the cell volume (surface density) of the proximal tubule wall and Fig. 5b shows the total area of lateral and basal cell membranes in one mm proximal tubule. Vertical bars indicate the standard error. The difference between groups I and II in Fig. 5 as well as the difference between groups I and II and groups II and III in Fig. 5b are statistically significant ($p < 0.05$).

et al. 1976, Larsson and Horster 1976). These studies showed similar ultrastructural and transporting characteristics of the proximal tubules as did the tubules in the present study (Larsson and Horster 1976). Furthermore, the specific activity of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ was fairly constant during the development (Horster *et al.* 1976) resulting in an increased amount of enzyme since the area of basal and lateral cell membranes increased. The parallel developmental pattern for the relative mitochondrial volume and fluid reabsorption related to the size of the lateral and basal cell membrane may reflect an increased energy requirement for the active sodium transport.

Previous studies performed on the *in vitro* perfused isolated rabbit proximal tubule have shown that the influence of transepithelial hydrostatic and oncotic pressure gradients on net volume flow is significantly higher in proximal tubules from 2- to 6-day-old rabbits than in proximal tubules from adult rabbits (Horster and Larsson 1976). Simultaneously performed ultrastructural studies using electron dense tracers showed that the proximal tubular epithelium from the postnatal rabbits is more leaky than that from adult rabbits. This indicates that the change in hydraulic conductance is due to alteration of the paracellular pathway. It seems very likely that this maturational change will also influence the development of transepithelial fluid transport in the rat proximal tubule. The resistance of the paracellular shunt will, however, vary with the state of hydration and will be

30- Relative volume of mitochondria in proximal tubule cell, %

Total mitochondrial volume in one mm PT μ^2

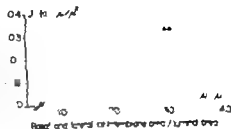


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Fig. 7 Correlation between proximal tubular fluid reabsorption related to the luminal area (J) (a) and to area of basal and lateral cell membranes related to the luminal area (excluding the area of the microvilli) during the postnatal development of the proximal tubule. Correlation coefficient (R)



studied the scatter of the values for tubular reabsorptive capacity was fairly small. This suggests that the reproducibility of the method used is satisfactory when applied to a homogeneous group of rats.

The question whether the method will allow comparison of results obtained from rats of different ages must, however, be considered. The reabsorptive rate of the tubules determined with shrinking drop technique has been found to be influenced by the geometry of the tubule (Györy 1971) and the intraluminal hydrostatic pressure (Grandchamp and Bostnapp 1972). The influence of the first factor will to a large extent be overcome if the reabsorptive rate is expressed per unit apparent tubular lumen area (Györy 1971). Since this was done in this study the change in tubular diameter that occurred during development still probably does not invalidate comparison between the groups.

The intraluminal hydrostatic pressure was not recorded. In order to make the experimentally induced pressure changes comparable among the different groups, however, the Gertz technique (Gertz 1973) was modified so that the tubule was only punctured once and the tips of the puncturing pipette were adapted to the size of the tubule. The fact that the quotient between the free flow and stop flow diameter was almost constant in the different age groups studied also suggest that the effect on intraluminal pressure of puncturing the tubule and injecting oil was comparable in all rats studied (Table II).

Part of this study was presented at the Vth International Congress of Nephrology in Florence, Italy 1975 (Abstract no 80). This study was supported by the Swedish Research Council Proj. no 876-19X-03644-058 and grant from the Karolinska Institute (Research grants). We thank Mrs Åsa-Christine Ekblom and Miss Åsa-Christine Ernsthjelt for excellent technical assistance.

References

- APPELLA, A. and P. HENRIK, Development of glomerular perfusion rate and nephron filtration rate in rats 17-60 days old. *Amer. J. Physiol.* 1973, 226, 1319-1323.
- APPELLA, A., O. BROMBERG, K. THOMASSEN and R. ZETTERSTROM, Renal response to an oral sodium load in newborn full-term infants. *Acta paediatr. (Uppsala)* 1972, 61, 670-676.
- APPELLA, A., O. BROMBERG, K. THOMASSEN and R. ZETTERSTROM, Renal control of sodium and fluid balance in newborn infants during intensive intensive therapy. *Acta paediatr. (Uppsala)* 1973, 62, 723-733.
- APPELLA, A., O. BROMBERG, K. THOMASSEN and R. ZETTERSTROM, Developmental or renal control of salt and fluid homeostasis during the first year of life. *Acta paediatr. (Uppsala)* 1973, 62, 391-398.
- BAKER, M., H. AYERMAN and E. W. WILSON, Effect on intraluminal bicarbonate and chloride on fluid absorption by the rat proximal tubule. *Kidney Int.* 1976, 9, 457-466.
- DIAMOND, J. The coupling of solute water transport in epithelia. In: *Renal transport and diuretics*. Edited by T. H. K. and H. J. J. Berlin, Springer-Verlag, 1969, p. 77.

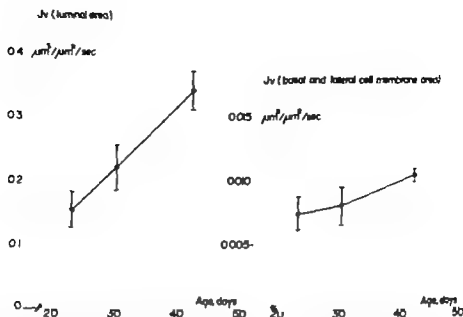


Fig. 6 Proximal tubular fluid reabsorption related to the luminal area (J_v (a)) (left) and fluid reabsorption to the area of lateral and basal cell membranes during postnatal development (right). Vertical bars indicate the standard error. The difference between groups I and II and between groups II and III (left) is statistically significant ($p < 0.01$) as well as the difference between groups II and III (right) ($p < 0.01$).

most pronounced during hydropenia (Grandchamp and Boulpaep 1974). Thus it has been assumed that during hydropenia the transepithelial sodium transport will even in leaky epithelia be the major determinant of proximal tubular water reabsorption occurring both in the cellular and paracellular pathways (Horster and Larsson 1976). It is therefore suggested that in the hydropenic immature animal, proximal tubular fluid reabsorption is more dependent by the availability of membrane bound active transporting enzyme for sodium than during hydration or extracellular volume expansion.

During the period between 22 and 44 days of rat postnatal development, the proximal tubular reabsorptive rate during hydropenia increased 2.2-fold. Assuming a constant diameter of the convoluted proximal tubule, the inner surface area of the total convoluted tubule would be $2\pi rL$, μm where r is the radius and L is the length of the proximal tubule. Using the values from Table I it is found that the surface area will increase 1.9-fold from 22 to 44 days of life. The filtered load in the superficial nephrons increases almost 7 fold, i.e. somewhat more than the product of J_v (a) and total lumen area. This indicates either an increased reabsorption per unit filtered load in the proximal tubule of the young rat or a non-homogenous development of the different parts of the proximal tubule.

The present study was performed after only four hours of food and fluid deprivation. Pilot studies showed that longer periods of food and fluid deprivation resulted in functionally unstable animals, in particular the younger animals. The low fractional water excretion in all age groups studied, which did not differ significantly from the findings in 40- to 45-day-old rats that were subjected to 12 hours food and fluid deprivation, does, however suggest that the animals were in a satisfactory stage of hydropenia. Within each group

Ventilatory and occlusion pressure response to CO₂ and hypoxia with resistive loads

By

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Abstract

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Steady-state responses to hyperoxic hypercapnia and eucapnic hypoxia were measured both as minute ventilation (\dot{V}_E) and as inspiratory mouth occlusion pressure (P_{10}) with and without 20 cm H₂O of added resistance (R). Reduction in slope of the ventilatory response to CO₂ with R was highly significant in all subjects. In contrast the response to hypoxia was barely significantly reduced in 1 subject and not significantly decreased in two. Although P_{10} was higher with than without R under all conditions, the slope of the P_{10} response to CO₂ with R was not increased in two subjects and only slightly increased in the third. The slope of the P_{10} response to hypoxia was significantly greater in all subjects with R. Expiratory reserve volume as measured with R but the change as the same with hypoxia and hypercapnia. We conclude that ventilation is better maintained with resistive loading during hypoxia than during hypercapnia and that this results from greater force output of expiratory muscles as reflected by higher P_{10} . This suggests greater neural output to these muscles.

An increase in airway resistance, whether with mechanical resistors (Cherniack and Siskind 1956) or with increased density of respired gases (Doell *et al.* 1973) is associated with a reduction in the ventilatory response to CO₂. There is less agreement about the effect of increased flow resistance on the ventilatory response to hypoxia. An earlier study in this laboratory (Barnett and Rasmussen 1970) showed that the ventilatory response to eucapnic hypoxia was reduced by adding external airway resistance. It appeared, however, that this effect of increased resistance was less marked than was the effect of the same added resistance on the ventilatory response to CO₂. There have been more recent reports describing a difference in the effect of resistive loading on the ventilatory responses to these two stimuli (Doell *et al.* 1973, Levson and Cherniack 1971). Others have emphasized the similarities between hypoxic and hypercapnic responses during loaded breathing (Rebock *et al.* 1975, Rebock and Juniper 1975).

If ventilation is indeed better maintained in the presence of resistive loads when the principal added stimulus is hypoxia then it might be predicted that the inspiratory force

- GERTZ, K. H. Transubulläre Natriumchloridflüsse und Permeabilität für nichtelektrolyte im proximalen und distalen Ko-volut der Rattenniere. *Pflügers Arch. ges. Physiol.* 1963 276, 336-356.
- GRANDCHAMP, A. and E. L. BOULPAEP. Effect of intraluminal pressure on proximal tubular sodium reabsorption. A shrinking drop micropuncture study. *Yale J. Biol. Med.* 1972 45 275-288.
- GRANDCHAMP, A. and E. L. BOULPAEP. Pressure control of sodium reabsorption and intercellular back flux across proximal kidney tubule. *J. clin. Invest.* 1974 54 69-82.
- GREEN, R. and G. GIERANT. Ionic requirements of proximal tubular sodium transport. I. Bicarbonate and chloride. *Amer. J. Physiol.* 1975 229 1216-1226.
- GYÖRY, A. Z., Reexamination of the split droplet method as applied to kidney tubules. *Pflügers Arch. ges. Physiol.* 1971 324 328-343.
- HILGER, H. H. J. D. KLÖPPER and K. J. ULLRICH. Wasserrückresorption und Ionentransport durch die Sammelrohrzellen der Säugetiermilch. *Pflügers Arch. ges. Physiol.* 1958 267 218-229.
- HORSTER, M. and L. LARSSON. Mechanisms of fluid absorption during proximal tubule development. *Kidney Int.* 1976, 10 348-363.
- HORSTER, M. and J. E. LEWY. Filtration fraction and extraction of PAH during neonatal period in the rat. *Amer. J. Physiol.* 1970, 219 1061-1065.
- HORSTER, M. and H. VALTIN. Postnatal development and renal function. Micropuncture and clearance studies in the dog. *J. clin. Invest.* 1971 50 779-793.
- HORSTER, M., L. LARSSON and U. SCHMIDT. Characteristics of the maturing proximal tubule studied *in vitro* on isolated perfused nephron segments. *Abstr. XI Smp. Gesellschaft für Nephrologie München* 1976.
- HUBER, C. G. On the development and shape of uriniferous tubules of certain of the higher mammals. *Amer. J. Anat.* 1905 4 suppl. 1 p. 1.
- KYTE, J. Immunoferritin determination of the distribution of (Na⁺ + K⁺) ATPase over the plasma membranes of renal convoluted tubules II: Proximal segment. *J. cell Biol.* 1976, 68 304-318.
- LANGER, K. H., W. THOMES and M. WIEDERHOLT. Licht- und elektronmikroskopische Untersuchungen an proximalen Tubulusconvolut der Rattenniere nach intraluminaler Ölojektion. *Pflügers Arch. ges. Physiol.* 1968 302 149-165.
- LARSSON, L., The ultrastructure of the developing proximal tubule in the rat kidney. *J. Ultrastruct. Res.* 1975, 51 119-139.
- LARSSON, L. and M. HORSTER. Ultrastructure and net fluid transport in isolated perfused developing proximal tubules. *J. Ultrastruct. Res.* 1976, 54 276-285.
- LEWY, J. E. and E. E. WINDHAGER. Peritubular control of proximal tubular fluid reabsorption in the rat kidney. *Amer. J. Physiol.* 1968 214 943-954.
- MAUNSBACH, A. B. The influence of different fixatives and fixation methods on the ultrastructure of rat kidney proximal tubule cells I. Comparison of different perfusion fixation methods and of glutaraldehyde formaldehyde and osmium tetroxide fixatives. *J. Ultrastruct. Res.* 1966 a, 15 242-282.
- MAUNSBACH, A. B., Observations on the segmentation of the proximal tubule in the rat kidney. Comparison of results from phase contrast fluorescence and electron microscopy. *J. Ultrastruct. Res.* 1966 b, 16 239-258.
- NAKANMA, K., J. R. CLAPP and R. R. ROBINSON. Limitations of the shrinkage-drop micropuncture technique. *Amer. J. Physiol.* 1970, 219 345-357.
- SACKIN, H. and E. BOULPAEP. Models for coupling of salt and water transport. Proximal tubule reabsorption in Necturus kidney. *J. Gen. Physiol.* 1975 66 671-733.
- SKOU, J. C., Enzymatic basis for active transport of Na⁺ and K⁺ across cell membranes. *Physiol. Rev.* 1965, 45 596-617.
- SPITZER, A. and M. BRANDER. Functional and morphologic maturation of the superficial nephros. Relationship to total kidney function. *J. clin. Invest.* 1974 53 279-287.
- WAHL, M. and J. SCHÖRMANN. Microdissection study of the length of different tubular segments of rat superficial nephrons. *A. Anat. Entwickl. Gesch.* 1969 129 128-134.
- WEINEL, E. R., G. S. KESTLER and W. R. SCHERLE. Practical stereological methods for morphometric cytology. *J. Cell Biol.* 1966, 30, 23-38.
- WINDHAGER, E. E. and G. GIERANT. Proximal sodium and fluid transport. *Kidney Int.* 1976, 9 121-131.

Ventilatory and occlusion pressure response to CO₂ and hypoxia with resistive loads

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Abstract

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Steady-state responses to hypercapnic hypercapnia and eucapnic hypoxia were measured both as minute ventilation (\dot{V}) and as inspiratory mouth occlusion pressure (P_{ao}) with and without 25 cm H₂O l/s added resistance (R). Reduction in slope of the ventilatory response to CO₂ with R was highly significant in all 3 subjects. While the response to hypoxia was barely significantly reduced in 1 subject and not significantly decreased in two. Although \dot{V} was higher with than without R under all conditions, the slope of the \dot{V} response to CO₂ with R was not increased in two subjects and only slightly increased in the third. The slope of the \dot{V} response to hypoxia was significantly greater in all subjects with R. Expiratory reserve volume was increased with R but the change as the same with hypoxia and hypercapnia. We conclude that ventilation is better maintained with resistive loading during hypoxia than during hypercapnia and that this results from greater force output of inspiratory muscles as reflected by a higher P_{ao} . This suggests greater neural output to these muscles.

An increase in airway resistance, whether with mechanical resistors (Cherniack and Snidal 1956) or with increased density of respired gases (Doell *et al.* 1973) is associated with a reduction in the ventilatory response to CO₂. There is less agreement about the effect of increased flow resistance on the ventilatory response to hypoxia. An earlier study in this laboratory (Barnett and Rasmussen 1970) showed that the ventilatory response to eucapnic hypoxia was reduced by adding external airway resistance. It appeared, however, that this effect of increased resistance was less marked than was the effect of the same added resistance on the ventilatory response to CO₂. There have been more recent reports describing a difference in the effect of resistive loading on the ventilatory responses to these two stimuli (Doell *et al.* 1973, Levison and Cherniack 1971). Others have emphasized the similarities between hypoxic and hypercapnic responses during loaded breathing (Rebuck *et al.* 1975, Rebuck and Juniper 1975).

If ventilation is indeed better maintained in the presence of resistive loads when the principal added stimulus is hypoxia then it might be predicted that the inspiratory force-

output response during resistive loading would be increased more by a hypoxic stimulus than by a hypercapnic stimulus which elicits the same increase in ventilation in the absence of added resistance ("equivalent stimulus"). To test this hypothesis the present study was designed to measure and compare the responses to hypoxia and to hypercapnia both in terms of minute ventilation (V_E) and in terms of mouth occlusion pressure, *i.e.* mouth pressure after the first 0.1 s of an occluded inspiration ($P_{0.1}$). This measurement reflects the force of approximately isometric contraction of inspiratory muscles and therefore the neural output to these muscles (Whitelaw *et al.* 1975).

Methods

3 healthy young adults well trained as subjects in respiratory experiments were used in this study. The 2 male subjects were 30 (TH) and 26 (HM) years old, the one female (BS) was 25 years old. For purposes of obtaining informed consent the experimental conditions were described to all subjects. They were not made aware of the aims of the experiments and were not told which type of experiment was to be done on a given day. These steady-state experiments were begun at 9 AM with the subject comfortably seated in a semirecumbent position. They read material of their own choice during the experiments. Hypoxic and hypercapnic gas mixtures were delivered from pre-mixed tanks via two flowmeters for adjustments of the final mixture and were humidified before collection in a bag reservoir for delivery to a low resistance two-way valve. Expired air was passed through a dry gasmeter or into Douglas bags for volume measurements and gas analysis.

All experiments were done in the same way and were of 4 types: hypoxia without added resistance (R), hypoxia with R, CO_2 without R, CO_2 with R. Only one type of experiment was done each day and consisted of 3 steady-state periods of 15–20 min separated by 5–10 min rest periods. The following sequence was used in all experiments: atmospheric air, first level of test gas, second level of test gas. Ventilation and end-tidal CO_2 were continuously monitored. Data points were established from measurements made during intervals of 2.5 min at the end of each steady state period. A total of 49 expts. of all types were performed. In all studies with hypoxia, CO_2 was included in the mixture to maintain a normal end-tidal CO_2 . Mean end-tidal CO_2 tensions (± 1 S.D.) in the 3 subjects were 39.39 (± 0.48), 39.41 (± 0.96) mmHg and 39.13 (± 0.60) with R and 39.69 (± 0.50), 39.45 (± 0.69) and 39.35 (± 0.57) without R. Experiments with hypercapnia were performed the same way with inspired CO_2 regulated to obtain steady-state hypoventilation at two levels approximating the two levels obtained with hypoxia. Alveolar O_2 tension was kept between 150 and 200 mmHg during the experiments with hypercapnia.

Resistive loading was with porous discs warmed to prevent condensation and interposed between the subject and the two-way valve thus imposing added resistance during both phases of the respiratory cycle. The resistance of the discs was 25 cm $\text{H}_2\text{O/l/s}$ and linear between 0.2 and 1.0 l/s. In hypoxic experiments with R were the alveolar Po_2 and end-tidal CO_2 (see above) adjusted to the same level as in experiments without R. From experience with our previous experiments it became possible to mix gases to obtain the desired ventilation and alveolar gas concentrations. Thereafter the experiments were done in random sequence over a period of approximately 8 months.

Inspired and expired gas samples were analyzed either by the micro Scholander method or with a Beckman Model LB-1 CO_2 analyzer and a Servomex oxygen analyzer. The latter instruments were calibrated with gases of known concentration. End-tidal CO_2 tension (P_{ETCO_2}) was calculated from the end of expiratory plateau of the record from Beckman infra red CO_2 analyzer. Sampling was carried out by micro sampler from the external airway distal to the resistor discs and corrections were made in V_E for gas removed through the pump. The CO_2 analyzer was calibrated with gases of known CO_2 concentrations immediately before and immediately after observation period. Using P_{ETCO_2} in the Bohr formula, as a substitute for P_{aCO_2} , dead space (V_D) was calculated. Assuming the same V_D for O_2 and CO_2 , alveolar oxygen tensions (P_{AO_2}) were calculated from the Bohr formula. All calibration gas mixtures were analyzed by the micro Scholander method. With simultaneous analysis of arterial and arterialized capillary blood along with end-tidal and alveolar gases, we have shown that the added resistance does not change alveolar-arterial Po_2 gradient or end-tidal-arterial P_{CO_2} gradient (Barnett and Rasmussen 1970).



Fig. 1. Ventilatory responses to CO_2 are shown in the plots on the left and responses to hypoxia on the right. Open circles represent measurements without added resistance, closed circles measurements with added resistance. PETO_2 levels during hypoxia are given in the text.

For purposes of continuous monitoring of ventilation, expired gas is passed through a dry gasmeter equipped with potentiometer. For final measurements of minute ventilation and collection of samples for analysis the expired gas was diverted into Douglas bags. All volumes are converted to STPD. Respiratory frequency was obtained from the CO_2 analyzer tracing.

Mouth occlusion pressure was measured in most experiments by a method described elsewhere (Ruzicewicz and Barnett 1976) in which a small lever is opened slowly during the expiratory phase allowing compressed air to advance a rubber ball into an opening which occludes the inspiratory airway. Pressure curves were obtained by recording the amplified output of a Statham PM5TC transducer on a storage oscilloscope. Occlusion time was limited to 0.25 sec since the occluder was automatically released by a circuit which was actuated by the negative pressure itself. This system is completely airtight. Air flow monitored with a Fleisch pneumotachometer placed between the mouth piece and the two-way valve assured that no flow occurred during occlusion. Mouth occlusion pressures were measured randomly 4 times during each measurement period of no less than 30 intervals. The inspiratory time was measured from the zero pressure points of three breaths just prior to each occluded breath.

Pressure, flow, CO_2 , and volume (from gasmeter) were recorded on an Elasm-Schönmader Mingograph recorder.

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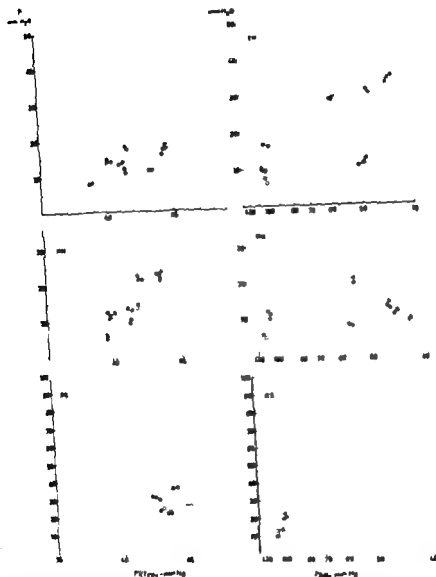


Fig. 2 Occlusion pressure responses to CO₂ (left) and to hypoxia (right). Open circles represent measurements without added resistance, closed circles measurement with added resistance.

normal atmospheric air the mean P_{oc} without loading in subjects TH, HM and BS was 34, 63, and 11.9 mmHg respectively. With the increase in airway resistance that was used in these studies, these mean values on atmospheric air were approximately doubled and the difference was highly significant. With hypercapnia the P_{oc} values also tended to be higher with than without resistance for any level of P_{atm} . With hypoxia P_{oc} was definitely higher with than without R at all levels of hypoxia. Linear regressions were calculated as

TABLE 1 Analysis of slopes of ventilatory responses to hypercapnia and hypoxia.

Subject	No added resistance		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef.	
$\Delta \dot{V}_E / \Delta P_{ETCO_2}$					
TH	1.409	0.935	0.537	0.740	<0.001
HM	1.503	0.870	0.477	0.809	<0.001
BS	1.110	0.879	0.585	0.924	<0.001
$\Delta \dot{V}_E / \Delta 1/(P_{AO_2} - 32)$					
TH	71.85	0.801	65.23	0.839	NS
HM	85.36	0.873	60.89	0.909	<0.05
BS	95.48	0.725	97.42	0.898	NS

Expiratory reserve volumes (ERV) as well as vital capacities (VC) were measured in an additional 5 expts. during hypoxia and 6 expts. during hypercapnia. For this purpose the subjects were in a closed displacement body plethysmograph with box gas volume changes being monitored with a Krogh spirometer. The box was tilted so that the subject was in a semi-reclining position similar to that used in the other experiments. After interposing the resistor the subjects were then allowed to breathe either hypoxic or hypercapnic mixtures for 15 min and measurements of P_{ETCO_2} , \dot{V}_E and mouth occlusion pressures were again made before and after the collection periods as described above. The final measurements were made 5 min after removal of the resistors but with the inspired gas remaining the same. In one experiment in each subject ERV was measured in a similar fashion while breathing air during hypoxia without resistor, and 10 min after the period of hypoxia.

Statistical tests were done using standard programs for CompuCorp 327 computer which gave mean \pm SD \pm SE, regression line coefficients a and b , and correlation coefficient r . Tests of significance of differences between regression lines were done by the method described by Hald (1952).

Results

The ventilatory responses to hyperoxia, hypercapnia and to eucapnic hypoxia in the three subjects are shown in Fig. 1. In order to facilitate visual comparison the 2 sets of data for each subject are placed together and the $\dot{V}_E - P_{\text{AO}_2}$ relationship has been plotted on semi-logarithmic paper with P_{AO_2} values decreasing from left to right. It is apparent that the ventilatory response to CO_2 was reduced in all subjects with resistive loading when compared with that in the unloaded state. When ventilation was plotted against P_{AO_2} , a reduction in the response in the presence of increased resistance was not so apparent. In Table 1 are shown the results of analyses of these data. The slopes and correlation coefficients for linear regressions of the relationship between P_{ETCO_2} and \dot{V}_E are shown. The reduction in slopes with increased airway resistance is highly significant in all subjects. In order to analyse the relationship between P_{AO_2} and \dot{V}_E as a linear function the term $1/(P_{\text{AO}_2} - 32)$ was used as suggested by Byrne-Quinn *et al* (1972). Analysis of \dot{V}_E versus this term shows no difference in the slopes in two subjects and the decrease in slope with added resistance in subject HM is barely significant.

Fig. 2 presents in a similar way the responses to hypercapnia and to hypoxia expressed as mouth occlusion pressure 0.1 s after beginning an occluded inspiration ($P_{0.1}$). As with the ventilatory response there was rather wide scatter of points. For all measurements on

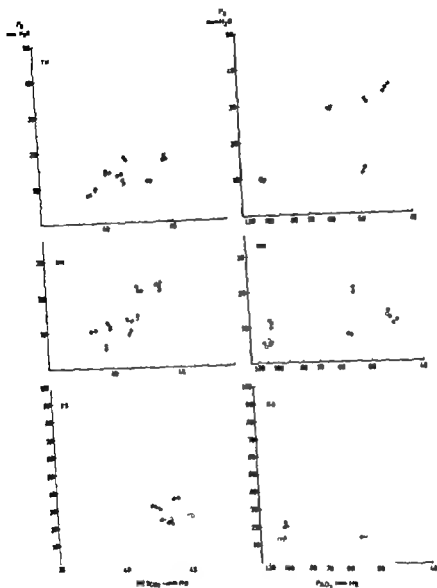


Fig. 2. Occlusion pressure responses to CO_2 (left) and to hypoxia (right). Open circles represent measurements without added resistance, closed circles measurements with added resistance.

normal atmospheric air the mean P_{oc} without loading in subjects TH, HM, and BS was 8.4, 6.3 and 11.9 mmHg respectively. With the increase in airway resistance that was used in these studies, these mean values on atmospheric air were approximately doubled and the difference was highly significant. With hypercapnia the P_{oc} values also tended to be higher with than without resistance for any level of P_{ICO_2} . With hypoxia P_{oc} was definitely higher with than without R at all levels of hypoxia. Linear regressions were calculated as

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Fig 2 Occlusion pressure responses to CO₂ (left) and to hypoxia (right). Open circles represent measurements without added resistance, closed circles measurements with added resistance

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TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{-1}/\Delta P_{ETCO_2}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_{-1}/\Delta I/(P_{A_{O_2}} - 32)$					
TH	81.00	0.697	13.40	0.668	<0.001
HM	77.69	0.681	167.00	0.852	<0.01
BS	176.90	0.701	912.70	0.904	<0.001

described above. Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e.* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO_2 breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of P_{ETCO_2} studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the P values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted.

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f). There was no significant difference in any subject between the slope of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance added f was always reduced but the relationship V_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3). In subjects BS there was an increase in both f and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_I) was always increased with added resistance. The increase varied from 45% to 61% above the T_I without a resistive load in subjects HM and TH and the difference was very similar with both hypercapnia and hypoxia. In subject BS the mean increase in T_I with loading in association with hypercapnia was 32% but with hypoxia it was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_I/T_T) was remarkably similar under all experimental conditions. In subjects TH and HM the T_I/T_T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40.8–53.9 Avg. = 46.5 mmHg) and in 6 expts. with hypercapnia

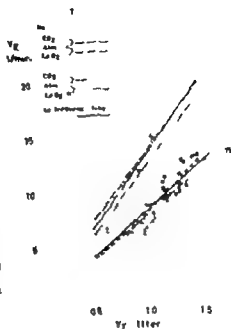


Fig. 3 Relationship between minute ventilation and tidal volume during hypercapnia and during hypoxia—with and without added airway resistance in subject TH.

Pressure, range 43.9–46.8, Avg. = 45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in each subject VC and ERV were measured before, during and after a 20 minute period of hypoxia ($P_{A_{CO_2}}$ 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output. The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$). Because of differences in the time required to reach maximum response for respectively hypoxia and hypercapnia, a steady-state method has been used for studying the responses as was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier series of experiments resistive loading led to some reduction in the ventilatory response to

TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P / \Delta P_{ETCO_2}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_0 / (\Delta P_{AO_2} - 32)$					
TH	81.00	0.697	213.40	0.668	<0.001
HM	77.69	0.681	167.00	0.852	<0.01
BS	176.90	0.701	912.70	0.904	<0.001

described above. Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e.* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

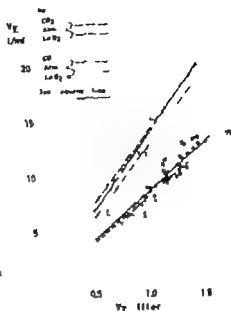
Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO₂ breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of P_{ETCO_2} studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the P values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted.

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f). There was no significant difference in any subject between the slope of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance added f was always reduced but the relationship V_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3). In subjects BS there was an increase in both f and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_i) was always increased with added resistance. The increase varied from 45% to 61% above the T_i without a resistive load in subjects HM and TH and this difference was very similar with both hypercapnia and hypoxia. In subject BS the mean increase in T_i with loading in association with hypercapnia was 32% but with hypoxia it was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_i/T) was remarkably similar under all experimental conditions. In subjects TH and HM the T_i/T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40.8–53.9 Avg. = 46.5 mmHg) and in 6 expts. with hypercapnia



1. Relationship between minute ventilation and P_T during hypoxia and during hypercapnia with and without added airway resistance in subject TH.

range 43.9–46.8 Avg. = 45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in each subject VC and ERV were measured before, during and after a 20 minute period of hypoxia (P_{AO_2} 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output.

The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$).

Because of differences in the time required to reach maximum response for respectively hypoxia and hypercapnia, a steady-state method has been used for studying the responses as was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier series of experiments resistive loading led to some reduction in the ventilatory response to

TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{12}/\Delta P_{ETCO_2}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_{12}/\Delta (P_{AO_2} - 32)$					
TH	81.00	0.697	213.40	0.668	<0.001
HM	77.69	0.681	167.00	0.852	<0.01
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described above. Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e.* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO₂ breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of P_{ETCO_2} studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the P_{ETCO_2} values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (\dot{V}_E) and of the timing of respiratory phases, the following observations were noted.

In the absence of a resistive load the increase in \dot{V}_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f). There was no significant difference in any subject between the slope of the relationship \dot{V}_E/V_T during hypercapnia from that during hypoxia. With resistance added f was always reduced but the relationship \dot{V}_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3). In subjects BS there was an increase in both \dot{V}_E and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_i) was always increased with added resistance. The increase was from 45% to 61% above the T_i without a resistive load in subjects HM and TH and the difference was very similar with both hypercapnia and hypoxia. In subject BS the increase in T_i with loading in association with hypercapnia was 32% but with hypoxia was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_i/T) was markedly similar under all experimental conditions. In subjects TH and HM the T_i/T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40.8–53.9 Avg. = 46.5 mmHg) and in 11 experiments with hypercapnia.

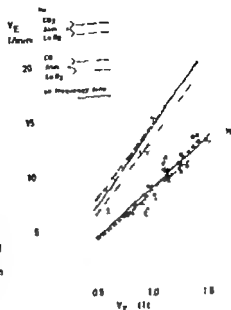


Fig. 3 Relationship between minute ventilation and tidal volume during hypoxia and during hypercapnia with and without added airway resistance in subject TH.

$\dot{V}_{E_{\text{max}}}$ range 43.9–46.2, Avg. = 45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in which subject VC and ERV were measured before, during and after a 20 minute period of hypoxia ($P_{A_{\text{CO}_2}}$ 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output. The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure (P_{ao}). Because of differences in the time required to reach maximum response for respectively hypoxia and hypercapnia, a steady-state method has been used for studying the responses as was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier series of experiments resistive loading led to some reduction in the ventilatory response to

TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{12}/\Delta P_{ETCO_2}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
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TH	81.00	0.697	213.40	0.668	<0.001
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described above. Testing of the difference in slope (Table II) shows what is apparent for the figures, i.e. the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

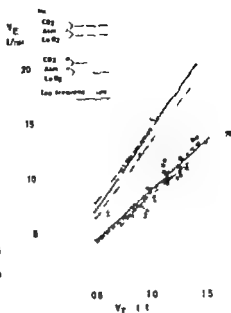
Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO₂ breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of P_{ETCO_2} studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the P_{12} values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted.

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (f). There was no significant difference in any subject between the slope of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance added f was always reduced but the relationship V_E/V_T was the same during hypoxia as hypercapnia in two of the subjects (Fig. 3). In subjects BS there was an increase in both f and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_I) was always increased with added resistance. The increase varied from 45% to 61% above the T_I without a resistive load in subjects HM and TH and this difference was very similar with both hypercapnia and hypoxia. In subject BS the increase in T_I with loading in association with hypercapnia was 32% but with hypoxia was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_I/T) was remarkably similar under all experimental conditions. In subjects TH and HM the T_I/T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{A_{O_2}}$ range 40.8–53.9 Avg. = 46.5 mmHg) and in 6 expts. with hypercapnia



Relationship between minute ventilation and volume during hypoxia and during hypercapnia with and without added airway resistance in TH.

range 43.9–46.8, Avg. = 45.1 mmHg). The ERV was always greater with than without resistance but there was no significant change in vital capacity. The magnitude change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in VC). It was the same with hypoxia as with hypercapnia in all subjects. In all instances RV returned to normal within 5 minutes after removal of the resistor even though the inhaled hypoxic or hypercapnic gas mixture remained the same. In one experiment in subject VC and ERV were measured before, during and after a 20 minute period of normoxia ($P_{A_{O_2}}$ 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

As the aim of this study to compare the effects of resistive loading on the responses to normoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses during mouth occlusion pressure which is a more meaningful indicator of neural output. Results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the loaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$). Because of differences in the time required to reach maximum response for respectively normoxia and hypercapnia, a steady-state method has been used for studying the responses as done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier experiments resistive loading led to some reduction in the ventilatory response to

TABLE II Analysis of slopes of occlusion pressure responses to hypercapnia and hypoxia.

Subject	No resistance added		Resistance added		P
	Slope	Corr Coef	Slope	Corr Coef	
$\Delta P_{\text{oc}}/\Delta P_{\text{ETCO}_2}$					
TH	1.307	0.830	0.799	0.480	NS
HM	1.193	0.697	1.540	0.668	NS
BS	2.720	0.690	5.220	0.837	<0.02
$\Delta P_{\text{oc}}/\Delta I/(P_{\text{A}_{\text{O}_2}} - 32)$					
TH	81.00	0.697	213.40	0.668	<0.001
HM	77.69	0.681	167.00	0.852	<0.01
BS	176.90	0.701	912.70	0.904	<0.001

described above. Testing of the difference in slope (Table II) shows what is apparent from the figures, *i.e.* the slopes are significantly greater with hypoxia and loading than those with hypoxia and no resistive load. In only one of the 3 subjects (BS) was this true during hypercapnia and even here the difference was smaller than during hypoxia.

Examination of the ventilatory response without added resistance (open circles in Fig. 1) reveals that the levels of hyperventilation attained with CO_2 breathing were approximately the same as with hypoxia. In Fig. 2 it is also apparent that without resistance mouth occlusion pressures over the range of P_{ETCO_2} studied were very similar to those over the range of hypoxia. This applied to all three subjects, although the *P* values were higher in subject BS under all conditions than in the two others.

When breathing patterns were examined by an analysis of the components of minute ventilation (V_E) and of the timing of respiratory phases, the following observations were noted.

In the absence of a resistive load the increase in V_E with hypercapnia and with hypoxia resulted almost entirely from an increase in tidal volume (V_T) with little change in respiratory frequency (*f*). There was no significant difference in any subject between the slope of the relationship V_E/V_T during hypercapnia from that during hypoxia. With resistance added *f* was always reduced but the relationship V_E/V_T was the same during hypoxia and hypercapnia in two of the subjects (Fig. 3). In subjects BS there was an increase in both *f* and V_T when hyperventilation resulted from hypoxia.

Inspiratory time (T_I) was always increased with added resistance. The increase varied from 45% to 61% above the T_I without a resistive load in subjects HM and TH and this difference was very similar with both hypercapnia and hypoxia. In subject BS the mean increase in T_I with loading in association with hypercapnia was 32% but with hypoxia it was only 10%. The ratio of inspiratory time to total respiratory cycle time (T_I/T) was remarkably similar under all experimental conditions. In subjects TH and HM the T_I/T ratios were slightly higher with than without resistance, but this difference was not significant.

Expiratory reserve volume (ERV) and vital capacity (VC) were measured in 5 experiments with hypoxia ($P_{\text{A}_{\text{O}_2}}$ range 40.8–53.9 Avg. = 46.5 mmHg) and in 6 expts. with hypercapnia

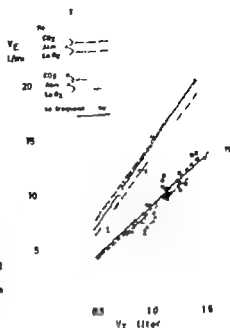


Fig. 1 Relationship between minute ventilation and tidal volume during hypoxia and during hypercapnia—each and without added airway resistance in subject TH

(P_{aO_2} , range 43.9–46.5, Avg. = 45.1 mmHg). The ERV was always greater with than without added resistance but there was no significant change in vital capacity. The magnitude of the change in ERV was not great (approximately 300 ml in subjects TH and BS and 400 ml in HM). It was the same with hypoxia as with hypercapnia in all subjects. In all instances the ERV returned to normal within 5 minutes after removal of the resistor even though the inspired hypoxic or hypercapnic gas mixture remained the same. In one experiment in each subject V_C and ERV were measured before, during and after a 20 minute period of hypoxia (P_{aO_2} , 43.3–46.5 mmHg). There was no change in the lung volume measurements.

Discussion

It was the aim of this study to compare the effects of resistive loading on the responses to hypoxia and hypercapnia. Since minute ventilation does not reflect neural output to inspiratory muscles in the presence of mechanical abnormalities, we have also measured responses in terms of mouth occlusion pressure which is a more meaningful indicator of neural output. The results show that in the presence of resistive loading ventilation was better maintained during hypoxia than during levels of hypercapnia which yielded similar responses in the unloaded state. This was accomplished by a relatively greater force output of the respiratory muscles as reflected in the inspiratory mouth occlusion pressure ($P_{0.1}$).

Because of differences in the time required to reach maximum response for respectively hypoxia and hypercapnia, a steady-state method has been used for studying the responses as was done earlier in similar experiments (Barnett and Rasmussen 1970). In the earlier series of experiments resistive loading led to some reduction in the ventilatory response to

eucapnic hypoxia but in all 3 subjects an appreciably greater reduction was seen in the response to normoxic hypercapnia. The resistor used in the present studies had a more linear pressure flow relationship and the resistance was less at higher flow rates, than the used in the earlier experiments. Only one (HM) of the 3 subjects in the recent experiments showed slight reduction in the ventilatory response to hypoxia with 25 cm H₂O/l/s added resistance. The response to hyperoxic hypercapnia, however, was significantly and markedly reduced in all subjects. Doell *et al* (1973) found no reduction in the ventilatory response to hypoxia in 6 subjects with a smaller increase in resistance. They did find a reduction in response to hypercapnic hypoxia at 4 ATA and Rebuck and Juniper (1975) described a reduced ventilatory response to hypercapnic hypoxia with 20 cm H₂O/l/s of inspiratory resistance. No direct comparison with normoxic or hyperoxic hypercapnia was reported. The results of these various experiments lead us to the conclusion that, whereas the hypoxic ventilatory response may obviously be reduced with sufficiently high resistive loads, ventilation is maintained at more nearly normal levels with moderate added resistance in association with hypoxia than when an equal resistive load is added during hypercapnia.

It was assumed earlier (Barnett and Rasmussen 1970) that a hypercapnic stimulus—and a hypoxic stimulus which resulted in similar ventilation were physiologically equivalent stimuli although it was recognized that alterations in resistance within the subject's airways as a result of one or both of these conditions might invalidate this assumption. In the present experiments without R the values for mouth occlusion pressure for any level of ventilation were identical regardless of whether the stimulus was hypoxia or hypercapnia. This observation makes it unlikely that differences in subjects' airways resistance were present. In so far as the P_{ao} is an indicator of the neural output to inspiratory muscles, it offers more convincing evidence that the two conditions were physiologically equivalent as respiratory stimuli.

Assuming that mouth occlusion pressure reflects the force output of inspiratory muscles, then the increment in this force was greater during hypoxia than during hypercapnia when external resistance was added. However the presumption that a greater occlusion pressure reflects a greater neural output to inspiratory muscles is not justified if end-tidal lung volume changes (Marshall 1962). In seated subjects Fitzgerald *et al* (1976) found variable but similar increases in FRC with short exposure to hypoxia (12% O₂) and hypercapnia (4% CO₂). Kellogg and Mines (1975) failed to demonstrate a change in FRC in seated or recumbent subjects when alveolar oxygen tensions were quickly lowered to 42–50 mmHg. The expiratory reserve volumes (ERV) were consistently increased in all 3 of our subjects in association with the resistive load, a finding which agrees with that reported by Zechman *et al* (1957). This change was not marked and was the same with hypoxia and hypercapnia. Furthermore, it was reversed by removal of the resistor while inspired gas concentrations remained unchanged. Comparable levels of hypoxia without added resistance failed to alter ERV. Earlier experiments on other normal subjects under almost identical conditions (Barnett, unpublished data) showed no effect of hypercapnia alone on these lung volumes. Thus, we were unable to explain the difference in effect of resistive loading on ventilatory and occlusion pressure responses with hypoxia and hypercapnia by differences in lung volumes. However it is possible that the thoraco-abdominal configuration and the diaphragmatic muscle length may have changed without alteration in the end-tidal volume.

The patterns of breathing during hypoxia and hypercapnia without added resistance were similar in all subjects, *i.e.* hyperventilation resulted mainly from an increase in tidal volume with little change in frequency. With resistive loading respiratory frequency was reduced and in 2 subjects (TG and HM) this effect was the same with hypoxia and hypercapnia, in one subject (BS) the increase in ventilation with hypoxia resulted from an increase in both frequency and tidal volume. We have reexamined data from 3 subjects previously reported (Barnett and Rasmussen 1970) and in all instances the relationship between \dot{V} and V was linear and similar during hypoxia to that during hypercapnia. Thus in 5 of 6 subjects the breathing patterns were as depicted in Fig. 3. The difference in the effect of resistive loads on the ventilatory and P_{aO_2} responses to hypoxia and hypercapnia, therefore, cannot be explained by difference in breathing patterns.

An increase in mouth occlusion pressure in association with inspiratory resistive loading was demonstrated by Kryger *et al.* (1975) and subsequently by Altose *et al.* (1976) and Kelsen *et al.* (1976). This increase was attributed to an increase in neural output. They suggest that this is mediated through a "higher" possibly cortical, mechanism since the increase in P_{aO_2} in goats with inspiratory loading during hypercapnia was almost entirely eliminated by sodium pentothal anesthesia (Isaza *et al.* 1976). Subsequently similar results have been reported in anesthetized human subjects (Whiteley *et al.* 1976). At present the exact nature of this increase in ventilatory drive in the presence of resistive loading is unknown. It would appear however that it is of greater magnitude with hypoxia than with hypercapnic stimulus which produces an equivalent ventilatory response in the absence of the resistive load. The data suggest a multiplicative interaction between hypoxia and resistive loading in determining the neural output to respiratory muscles. This situation is reminiscent of that which occurs with exercise where in both animals (Kao *et al.* 1967) and man (Astrand 1967) the exercise stimulus to ventilation has been found to interact with hypoxia in more than an additive manner. Breathing against resistance means that the respiratory muscles are in a state of exercise. It seems likely that these "exercising" muscles would influence ventilation as does muscular exercise otherwise and that this influence might be greater during hypoxia than during hypercapnia. Flewley *et al.* (1970) found normal or increased ventilatory responses to hypoxia in 7 of 9 patients with chronic obstructive disease (COPD) who showed reduced responses to CO₂. The results of the present study may contribute to the understanding of these findings and may help to explain the importance of the hypoxic stimulus in maintaining ventilation in the presence of obstructive airways disease.

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References

- ALTOSÉ, M. D., S. G. KELSEN, N. N. STANLEY, R. S. LEVISON, III, S. OBERNICK and A. P. FISHMAN. Effects of hypercapnia on mouth pressure during airway occlusion in conscious man. *J. appl. Physiol.* 1976, 40: 334-344.
- ANDERSON, E. Exercise and the regulation of ventilation. *Cerebral Res.* 1967, 20-21 (Suppl.), 132-145.
- BARNETT, T. B. and B. RASMUSSEN. Ventilatory responses to hypoxia and hypercapnia with external airway resistance. *Acta Physiol. Scand.* 1970, 80: 538-441.

- BYRNE-QUINN E., I. E. SODAL and J. V. WEIL, Hypoxic and hypercapnic ventilatory drives in child native to high altitude. *J appl. Physiol.* 1972. 32 44-46.
- CHEERNIAK, R. M. and D. P. SYDAL, The effect of obstruction to breathing on the ventilatory response to CO_2 . *J. clin. Invest.* 1956. 35 1286-1290.
- DEJOURS, P., Y. LARROUSSE and A. TEILLAC, Étude du stimulus ventilatoire proprioceptif mis en jeu par l'activité motrice chez l'Homme. *C. R. Acad. Sci. (Paris)* 1959. 248 2129-2131.
- DOELL, D. M. ZUTTER and N. R. ANTHONISEN, Ventilatory responses to hypercapnia and hypoxia at 1 and 4 ATA. *Respir. Physiol.* 1973. 18 338-345.
- FITZGERALD, R. S., F. GAMFINKEL, E. SILBERGELD and S. C. LOSCUTOFF, Factors in the interpretation of mouth occlusion pressure during measurement of chemosensitivity. *Chest* 1976. 70 Suppl. 145-149.
- FLEMLEY D. C., D. H. FRANKLIN and J. S. MILLAR, The hypoxic drive to breathing in chronic bronchitis and emphysema. *Clin. Sci.* 1970. 38 503-518.
- HALD, A. *Statistical Theory with Engineering Applications*. John Wiley & Son New York 1952. 571-573.
- ISAZA, G. B., J. D. POPPER, M. D. ALTORF, S. G. KILLEN and N. S. CHEERNIAK, Airway occlusion pressure in awake and anesthetized goats. *Respir. Physiol.* 1976. 27 87-98.
- KAO, F. F., S. LAHIRI, C. WANG and S. E. MEI, Ventilation and cardiac output in exercise-interaction of chemical and work stimuli. *Circulator Res.* 1967. 20-21 Suppl. I. 170-191.
- KELLOGG, R. H. and A. H. MIDER, Acute hypoxia fails to affect FRC in man. (Abstract) *Physiologist* 1975. 18 275.
- KILLEN, S. G., M. D. ALTORF, N. N. STANLEY, R. S. LEVISON, N. S. CHEERNIAK and A. P. FISHER, Effect of hypoxia on the pressure developed by inspiratory muscles during airway occlusion. *J. appl. Physiol.* 1976. 40 372-378.
- KRYGER, M. H., O. YACOB and N. R. ANTHONISEN, Effect of respiratory resistance on occlusion pressure in hypoxia and hypercapnia. *Respir. Physiol.* 1975. 24 241-48.
- LEVISON, H. and R. M. CHEERNIAK, Interdependence between mechanical resistances to breathing and the ventilatory response to hypercapnia and hypoxia (Abstract). *Clin. Res.* 1971. 19 803.
- MARSHALL, R., Relationship between stimulus and work of breathing at different lung volumes. *J. appl. Physiol.* 1962. 17 917-921.
- RASMUSSEN, B. and T. B. BARNETT, A new silent apparatus for measurements of mouth occlusion pressure (Abstract) *J. physiol. scand.* 1976. Suppl. 440. 181.
- REBUCK, A. S. and E. F. JUNGHER, Effect of resistive loading on ventilatory response to hypoxia. *J. appl. Physiol.* 1975. 38 965-968.
- REBUCK, A. S., M. BITTS and N. A. SAUNDERS, Effect of elastic loading on ventilatory response to hypoxia in conscious man. *J. appl. Physiol.* 1975. 39 548-551.
- WHITELAW, W. A., J. P. DERRIDON, J. COUTURE and J. MILIC-EMILI, Adaptation of anesthetized man to breathing through an inspiratory resistor. *J. appl. Physiol.* 1976. 41 285-291.
- WHITELAW, W. A., J. P. DERRIDON and J. MILIC-EMILI, Occlusion pressure as a measure of respiratory center output in conscious man. *Respir. Physiol.* 1975. 23 181-199.
- ZICHMAN, F. F., G. HALL and W. G. HALL, Effects of graded resistance to tracheal air flow in man. *J. appl. Physiol.* 1957. 10. 356-362.

Nervous control of pancreatic exocrine secretion in pigs

By

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Abstract

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The pancreatic secretion of fluid, bicarbonate and protein in response to electrical stimulation of the vagus and splanchnic nerves, to exogenous and endogenous secretin and to various pharmacological agents was studied in anesthetized young pigs (21 kg). Vagal stimulation increased flow, bicarbonate output and protein output in a frequency dependent manner: the half maximal effective frequency was 2-4 Hz and the maximal effective frequency 12 Hz. The secretory response to vagal stimulation was potentiated by physiological elevations of the arterial concentration of secretin brought about by injection of secretin or by acidification of the duodenal bulb. Simultaneous stimulation of the splanchnic nerves strongly inhibited the response to vagal stimulation; splanchnic nerve stimulation alone had no demonstrable effect. The flow and bicarbonate response to vagal stimulation was unaffected by atropine, but abolished by hexamethonium. Protein output was strongly inhibited by both agents. The response to intrarterial infusions of acetylcholine resembled that elicited by vagal stimulation but it was smaller and it was completely abolished by atropine and unaffected by hexamethonium. Alpha- and beta-adrenergic blockade stimulated rather than inhibited the secretory response to vagal stimulation. The portal vein plasma concentration of secretin was not affected by vagal stimulation. The results indicate that the protein response, and the flow and bicarbonate response to vagal stimulation are not brought about by the autonomic nervous system. An increased release of secretin is not involved. Peptidergic (VIP-containing) nerves may contribute.

The control of pancreatic secretion of fluid and bicarbonate is generally believed to be exerted chiefly by changes in the concentration of circulating secretin (Harper 1972). Radio-immunological determination of secretin concentrations in plasma have confirmed, that stimulation of acid into the small intestine is associated with increasing secretin concentrations, and that the resulting secretin concentrations when reproduced by exogenous secretin administration may explain a major part of the pancreatic secretory response to acidification (Schaffalitzky de Muckadell *et al.* 1977a, Rhodes *et al.* 1976, Isenberg *et al.* 1977, Häcki *et al.* 1977, Schaffalitzky de Muckadell *et al.* 1978).

It has also recently been established that the gastric emptying of acid into the duodenum and hence the duodenal pH are closely related to the concentrations of immunoreactive secretin in peripheral blood (Schaffalitzky de Muckadell and Fahreniérug (in press)). Ingestion of meal, however, is associated with only small and inconstant increases in peripheral

concentrations of secretin in man (Schaffalitzky de Muckadell and Fahrenkrug (in press Rhodes *et al* 1976 Fahrenkrug and Schaffalitzky de Muckadell 1977) although the pancreas in this situation would be expected to contribute to the digestive processes by a great increased production of juice, bicarbonate and enzymes (the dimension of the fluid response in man remains undocumented but in dogs there is a pronounced fluid response to feeding (Llanos *et al* 1977). If such a secretion takes place, it could not be due to the small rise in the secretion of secretin alone (Schaffalitzky de Muckadell *et al* 1978 Llanos *et al* 1977). The pancreas of man and pig has been reported to receive extensive cholinergic innervation (Comline *et al* 1964), and the pig has been reported to respond to vagal stimulation with profuse exocrine secretion (Hickson 1970 b). In other species, the volume and bicarbonate response to vagal stimulation is usually small (Thomas 1967 Preshaw 1967 Sjödén 1977) and other investigators were not able to confirm the above mentioned impressive fluid and bicarbonate response in pigs (Magee and White 1965).

We therefore decided to investigate, in anaesthetized pigs, the role of the autonomic nervous system in the control of pancreatic exocrine secretion and have attempted to identify the mechanisms by which this control is exerted.

Using the recently developed radioimmunoassay for secretin, we also evaluated the possible role of this hormone in the pancreatic response to vagal stimulation.

Materials and methods

I. Animal and operative procedure

50 kg female Danish Landrace pigs (weight 17–25 kg, median 1 kg) were obtained from the swine breeder Food but not water which contained 10% glucose was withdrawn 18 h before experiment.

Azaperone (Sedaperone Jansen Pharmaceutica Beerse Belgium) 4 mg/kg intramuscularly was used for premedication. Anesthesia was induced with halothane/N₂O/O₂ and maintained with chloralose 70 mg/kg intravenously. After insertion of an endotracheal cuffed tube intermittent positive pressure ventilation was maintained by a Bennett neopneuma ventilator in a semi-open system. Polyethylene catheters were inserted into the left femoral artery and vein. Intraarterial blood pressure and rectal temperature was continuously monitored. The animal were kept warm by means of an electric heating pad. The stomach, the duodenum, and the pancreas were exposed through mid-line incision. A polyethylene catheter (dead space 0.2 ml) was inserted into the pancreatic duct close to its entrance to the duodenum. The gastro-pyloric junction was closed by means of a ligature around the pylorus, carefully placed a closure in the muscular wall as possible to avoid damage to vessels and nerves. The gastric contents were continuously drained by an oro-gastric tube (Ch 30).

II. Experimental protocol

a) *Basal secretion* Pancreatic juice was collected for 15 min periods beginning 60 minutes after the completion of the operative procedure.

b) *Pancreatic exocrine response to secretin* 39 pigs received pure natural porcine secretin (GIII Research Laboratory Karolinska Institute, Stockholm, Sweden) 0.3 Clinical Units/kg i.v. as a bolus. This dose produced for more than 5 min plasma concentrations of secretin above that eliciting maximal secretion of fluid and bicarbonate (Schaffalitzky de Muckadell *et al* 1977). Pancreatic juice was collected for three 5-min periods, and a subsequent 15-min period. In the first 15 min period was collected for 5 min, 25–30 min after the secretin injection. In 10 pigs blood samples for secretin determination were drawn from the portal vein 30 min after the administration of secretin.

c) *Vagal stimulation* After thoracotomy the vagal trunks were identified above the diaphragm, and cut below the heart. The peripheral cut ends were threaded through a bipolar platinum electrode (Dand and Henderson 1967).

Stimulation was performed with constant current square-wave impulses (impulse duration 4 ms, 8 mA).

or 5 min (delivered by an Electronic Square-wave Stimulator Palmer U.K.). Applied frequency was varied between 0.25 and 40 Hz. Pancreatic juice was collected for three 5-min periods during and after the stimulation, when secretin had previously been administered at least 60 min elapsed before stimulation. In 25 expts. in 17 pigs blood samples for secretin determination are obtained from the portal vein at 15, 10, 5, 0, $\frac{1}{2}$, 1, 3, 5, 10, and 15 min after the start of stimulation. In 2 expts. blood samples were also obtained from femoral artery during vagal stimulation.

(4) *Stimulation by secretin of the response to vagal stimulation.* (1) Exogenous secretin. Vagal stimulation was carried out 30 min after the administration of secretin as described in section b, and compared to similar stimulations more than 60 min after secretin.

(2) Endogenous secretin. In 4 pigs vagal stimulation was performed during simultaneous perfusion of the duodenal bulb with acid. Two soft catheters (Ch. 18) were mounted in rubber stopper which was introduced into the duodenum through small distal duodenostomy and anchored 3 cm from the pylorus by means of ligature. The duodenal bulb was then perfused with 1) citrate buffer, 60.4 mmol/l and adjusted to pH 4 with HCl, and 2) citrate buffer 5 mmol/l, adjusted to pH 1 with HCl. Both buffers are made isotonic with NaCl, and heated to 37°C before perfusion. By titration to pH 7 both buffers were found to have titratable acidity of 100 mmol/l. Perfusion was carried out at flow of 30 ml/min, which was maintained for 15 min. After 20 min the vagus nerves were stimulated at 4 Hz. Pancreatic juice was collected for two 10 min periods and one 5 min period. Blood samples for secretin determination are obtained as described in section a.

(3) *The splanchnic nerves.* (1) The splanchnic nerves are cut just below the diaphragm in all expts. (see section a-g) except 5 in which the splanchnic nerves were dissected free; ligature was placed loosely around the nerves, and threaded through stiff tube which is exteriorized through the flanks of the animal. The vagal stimulation as carried out as above, and repeated after the splanchnic nerves had been severed by traction of the ligature. Pancreatic juice was collected as described in section c.

(2) *Splanchnic nerve stimulation.* The left splanchnic nerves are dissected free just below the diaphragm, cut, and the distal end threaded through bipolar platinum electrode. Stimulation was carried out using second stimulator (upulse duration 1 ms, 5 mA, 10 Hz for 3 min). The right splanchnic nerves are cut. Pancreatic juice was collected as described in section c.

(3) *Reproducibility and frequency of response.* In 7 pigs 5 min vagal stimulation were repeated 2-4 times over 3 h. In 6 pigs vagal stimulation was continued for 30 min.

(4) *Pharmacological experiments.* The pancreatic response to vagal stimulation was studied after blockade with atropine at various doses (0.005 to 2 mg/kg b.wt., as atropine sulphate). Atropine is also administered as placebo infusion (0.5 mg/kg initially followed by 1 mg kg⁻¹ h⁻¹) in 2 expts. Hexamethonium (Sigma Chemical Company St. Louis, Mo., U.S.A.) was given at 10-30 mg kg⁻¹ (as hexamethonium bromide). Propionyl (Lidral[®] 1 C.I. U.K.) was given as dose of 1 mg kg⁻¹. Flortoxybenzamine (Alfred Berzon, Copenhagen) was given as slow infusion, in total dose of 1 mg kg⁻¹. For studies with acetylcholine (Sigma Chemical Company), catheter was introduced via the right femoral artery into the aorta 10 cm up 5 cm above the outlet of the celiac artery. The acetylcholine was dissolved immediately before use in saline and infused by means of constant infusion syringe pump (Umax, Braun-Melsungen, GFR). Acetylcholine is infused for 5 min, and pancreatic juice was collected as during vagal stimulation (section c). In two pigs step-dose experiment was carried out, with doses ranging from 0.05 to 100 mg/h for 5 min. Pancreatic juice was collected for 5 min periods.

Laboratory analysis

Volume of the pancreatic juice was recorded to the nearest 0.01 ml. Bicarbonate and protein concentrations were determined as previously described (Schaffhauzky de Muckadeil *et al.* 1977).

Blood samples for secretin determination were drawn into chilled tubes containing heparin and aprotinin (Trasyol, Bayer GFR) 500 KIU/ml blood. Plasma secretin concentrations were determined radioimmunochemically (Schaffhauzky de Muckadeil *et al.* 1977a, Schaffhauzky de Muckadeil and Fakrekrug 1977c).

Calculations

The results concerning choledoch secretion are processed as follows. Flow of juice ml h⁻¹, concentration of protein in pancreatic juice g/l, output of protein g h⁻¹, concentration of bicarbonate mmol/l and output of bicarbonate mmol h⁻¹. The data are presented as the means followed by the 95% confidence limits of the means, or the total range for $n < 8$ (Dixon and Luntz 1968). Where two responses from the

same animal are compared the results are expressed in % of the previous response, and medians as above. Statistical evaluation was performed using the Mann-Whitney U-test, The Wilcoxon matched-pairs signed-ranks test, and The Friedman two-way analysis of variance (Siegel 1956).

Results

a) Basal secretion

There was a small basal secretion of juice in all animals, but the volume secreted was often too low compared to the dead space of the pancreatic catheter to allow bicarbonate and protein determination. Basal flow was recorded for 21 animals, and protein and bicarbonate concentrations determined in 8 samples. The results appear in Table I.

b) Pancreatic exocrine response to exogenous secretin

(Table I) An increased flow of juice was noted 30 to 40 s after the administration of secretin in all pigs. Stimulated secretion was maintained for at least 10 min. Compared to basal levels, flow was increased 102 times and bicarbonate concentration rose from 69 mmol/l to 143 mmol/l. The concentration of protein fell rapidly from the basal level of 8.7 g/l to 0.4 g/l, whereas protein output increased during the first 5 min period and then gradually fell below basal output. Twentyfive to 30 min after administration of secretin the flow of juice was still slightly but significantly elevated.

c) Vagal stimulation

The results of maximal or supramaximal stimulations (12–20 Hz) in 29 pigs are listed in Table II. The flow of juice increased 40–50 s after the start of stimulation in all pigs. Increased secretion could be maintained for as long as the electrical stimulation was continued (Table IX) but the secretion declined rapidly after cessation of stimulation and approached basal levels (or less) after 10 min. During the first 5 min of stimulation the flow of juice

TABLE I. Pancreatic exocrine response to secretin.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40–0.92) n = 21	8.7 (4.0–29.6) n = 8	69 (45–110) n = 8	0.011 (0.009–0.053) n = 8	0.080 (0.032–0.23) n = 8
Response to bolus injection of 0.5 CU/kg at 0 min (n = 39)					
0–5 min	57 (48–66)	5.9 (4.1–10.9)	131 (123–134)	0.29 (0.23–0.46)	7.6 (6.1–9.0)
5–10 min	31 (48–60)	0.4 (0.4–0.6)	138 (136–144)	0.024 (0.019–0.034)	7.1 (6.7–8.8)
10–15 min	36 (30–42)	0.4 (0.2–0.5)	143 (142–146)	0.009 (0.006–0.016)	5.4 (4.3–6.5)
15–30 min	9 (8–12)	0.5 (0.2–0.7)	130 (126–134)	0.004 (0.002–0.006)	1.07 (0.99–1.61)
25–30 min	2.1 (0.5–3.6) ^a	—	—	—	—

The data are presented as the median followed by the 95% confidence limits of the median.
This time interval was studied in 8 pigs only.

TABLE II. Pancreatic exocrine response to 5 min supramaximal vagal stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Basal secretion	0.56 (0.40-0.92) 21	2.7 (1.0-29.4) 8	69 (45-110) -8	0.011 (0.009-0.053) -8	0.080 (0.012-0.23) -8
1 min after start of stimulation					
1 (25)					
0-5	31 (24-42)	108 (90-140)	126 (120-152)	3.28 (2.30-3.87)	3.96 (2.45-5.46)
5-10	5.4 (3.9-6.6)	37 (25-61)	136 (128-140)	0.20 (0.15-0.24)	0.76 (0.59-0.99)
10-15	0.020 (0-0.120)	—	—	—	—

The data are presented as the median followed by the 95% confidence limits of the median.

increased 52 times, the concentration of protein 12 times, the bicarbonate concentration 1.8 times and the output of protein and bicarbonate 300 and 50 times, respectively.

In 23 animals the exocrine response to both exogenous secretin and to vagal stimulation was studied. Thus the response to vagal stimulations (which were performed more than 60 min after the injection of secretin at which time the plasma concentration of secretin had returned to the preinjection level (not shown)) could be compared to the response to secretin alone with each animal as its own control. The flow of juice during supramaximal vagal stimulation amounted to 59% (median, 95% confidence limits 47-75%) of that obtained in the second 5 min period after secretin. The bicarbonate output constituted 55% (42-75%) while the protein output during vagal stimulation was 9-100% (6.900-20.200%) of that obtained in the second 5 min period after secretin. In 4 of 23 animals the flow as well as the bicarbonate output exceeded that obtained after secretin, and in 7 and 9 respectively the flow and bicarbonate output constituted more than 70% of that obtained after secretin.

TABLE III. Pancreatic exocrine response to vagal stimulation at various frequencies.

Frequency Hz ^a	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
0.25 (1)	1.2	—	—	—	—
0.575 (1)	4.8	2.7	45	0.13	0.22
0.5 (2)	2.1, 0.46	—	—	—	—
2 (8)	14.1 (5.4-44.0)	118 (38-217)	111 (62-134)	1.37 (0.38-5.70)	1.31 (0.43-6.2)
4 (11)	18.6 (12.0-26.4)	120 (70-189)	110 (96-124)	2.30 (1.39-3.73)	1.90 (1.33-3.22)
8 (16)	31.0 (25-49)	116 (89-162)	124 (112-130)	3.96 (2.41-6.15)	3.90 (2.72-6.10)
12 (8)	43.3 (16.0-74)	101 (54-138)	133 (110-140)	3.65 (2.01-5.96)	5.62 (2.16-9.97)
20 (22)	27.0 (18.2-37.0)	106 (90-140)	124 (118-132)	2.14 (2.18-3.61)	3.26 (2.16-4.97)
40 (1)	24.0	46	124	1.10	2.37

^a Vagal stimulation was carried out for 5 min. The values are derived from analysis of the secretory response during these 5 min. Number of pigs in brackets in the first column. The data are presented as the median followed by the 95% confidence limits of the median. The effect of 4 Hz stimulation was studied in 11 pigs, at 5 pigs.

same animal are compared, the results are expressed in % of the previous response and as medians as above. Statistical evaluation was performed using the Mann-Whitney U test, The Wilcoxon matched-pairs signed-ranks test, and The Friedman two-way analysis of variance (Siegel 1956).

Results

a) Basal secretion

There was a small basal secretion of juice in all animals, but the volume secreted was often too low compared to the dead space of the pancreatic catheter to allow bicarbonate and protein determination. Basal flow was recorded for 21 animals, and protein and bicarbonate concentrations determined in 8 samples. The results appear in Table I.

b) Pancreatic exocrine response to exogenous secretin

(Table I) An increased flow of juice was noted 30 to 40 s after the administration of secretin in all pigs. Stimulated secretion was maintained for at least 10 min. Compared to basal levels, flow was increased 102 times and bicarbonate concentration rose from 68 mmol/l to 143 mmol/l. The concentration of protein fell rapidly from the basal level of 8.7 g/l to 0.4 g/l whereas protein output increased during the first 5 min period and then gradually fell below basal output. Twentyfive to 30 min after administration of secretin the flow of juice was still slightly but significantly elevated.

c) Vagal stimulation

The results of maximal or supramaximal stimulations (12–20 Hz) in 29 pigs are listed in Table II. The flow of juice increased 40–50 s after the start of stimulation in all pigs. Increased secretion could be maintained for as long as the electrical stimulation was continued (Table IX) but the secretion declined rapidly after cessation of stimulation and approached basal levels (or less) after 10 min. During the first 5 min of stimulation the flow of juice

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Response to bolus injection of 0.5 CU/kg at 0 min (n = 39)					
0–5 min	57 (48–66)	5.9 (4.1–10.9)	131 (123–134)	0.29 (0.23–0.46)	7.6 (6.1–9.0)
5–10 min	53 (48–60)	0.4 (0.4–0.6)	138 (136–144)	0.024 (0.019–0.034)	7.1 (6.7–8.8)
10–15 min	36 (30–42)	0.4 (0.2–0.5)	143 (142–146)	0.009 (0.006–0.016)	5.4 (4.3–6.5)
15–30 min	9 (8–12)	0.5 (0.2–0.7)	130 (126–134)	0.004 (0.002–0.006)	1.07 (0.99–1.61)
25–30 min	2.1 (0.5–3.6) ^a	—	—	—	—

The data are presented as the median followed by the 95% confidence limits of the median.
This time interval was studied in 8 pigs only.

TABLE II. Pancreatic exocrine response to 5 min supramaximal vagal stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
basal secretion	0.54 (0.40-0.97) 21	8.7 (4.0-29.6) —§	69 (45-110) §	0.011 (0.009-0.053) —§	0.080 (0.032-0.23) —§
5 min after start of stimulation (2§)					
1-5	31 (24-42)	108 (90-140)	126 (120-132)	3.28 (2.30-3.65)	3.96 (2.45-5.46)
5-10	5.4 (3.9-6.4)	37 (25-61)	136 (128-140)	0.20 (0.15-0.24)	0.76 (0.59-0.89)
1-15	0.020 (0-0.120)	—	—	—	—

§ The data are presented as the median followed by the 95% confidence limits of the median.

increased 52 times, the concentration of protein 12 times, the bicarbonate concentration 1.8 times and the output of protein and bicarbonate 300 and 50 times, respectively.

In 11 animals the exocrine response to both exogenous secretin and to vagal stimulation was studied. Then the response to vagal stimulation (which were performed more than 50 min after the injection of secretin at which time the plasma concentration of secretin had returned to the preinjection level (not shown)) could be compared to the response to secretin alone with each animal as its own control. The flow of juice during supramaximal vagal stimulation amounted to 59% (median, 95% confidence limits 47-75%) of that obtained in the second 5 min period after secretin. The bicarbonate output constituted 55% (42-75%) while the protein output during vagal stimulation was 9-100% (6.900-20.200%) of that obtained in the second 5 min period after secretin. In 4 of 23 animals the flow as well as the bicarbonate output exceeded that obtained after secretin, and in 7 and 9 respectively the flow and bicarbonate output constituted more than 70% of that obtained after secretin.

TABLE III. Pancreatic exocrine response to vagal stimulation at various frequencies.

Frequency Hz ^a	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
0.25 (1)	1.2	—	—	—	—
0.375 (1)	4.8	2.7	45	0.13	0.22
0.5 (2)	2.1 (0.66)	—	—	—	—
2 (6)	14.1 (5.4-46.0)	118 (38-217)	85 (62-134)	1.57 (0.38-5.70)	1.31 (0.43-6.2)
4 (11)	18.6 (12.0-26.4)	120 (70-189)	110 (96-124)	2.30 (1.19-3.73)	1.90 (1.32-3.22)
8 (14)	31.0 (25-49)	116 (69-162)	124 (112-130)	3.96 (2.41-6.15)	3.90 (2.72-6.10)
12 (8)	43.5 (18.0-74)	101 (54-198)	133 (110-140)	3.63 (2.01-5.94)	5.62 (2.16-9.97)
20 (22)	27.0 (19.2-37.0)	108 (90-140)	124 (118-132)	3.14 (2.18-3.61)	3.26 (2.16-4.97)
40 (3)	24.0	46	124	1.10	2.97

^a Vagal stimulation was carried out for 5 min. The values are derived from analysis of the secretory responses during these 5 min. Number of pigs in brackets in the first column. The data are presented as the medians followed by the 95% confidence limits of the median. The effect of 4 Hz stimulation was studied in 11 experiments, 3 pigs.

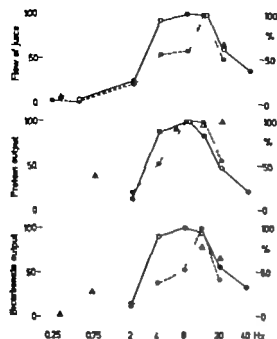


Fig. 1 Individual values for flow of juice, protein output, and bicarbonate output expressed as per cent of the maximum values obtained during vagal stimulation at various frequencies in three pigs.

In another series of expts. the responses to various frequencies of stimulation were studied (Table III). In 3 out of 4 expts. with frequencies below 1 Hz, the flow increased during stimulation. The half-maximal effective frequency was between 2 and 4 Hz and maximal stimulation of flow occurred at 12 Hz. Higher frequencies appeared less effective. The maximum concentration of protein was obtained with 2 Hz, while maximal bicarbonate output occurred at 12 Hz.

In 10 pigs 3-4 frequencies were used in the same pig. In 3 pigs 6-7 different frequencies were studied. In both cases 5 min periods of stimulation and intervening intervals of 10 min were used. The individual values for flow, protein output and bicarbonate output expressed as per cent of the maximum values obtained, are shown in Fig. 1. Although differing in sensitivity frequency-dependent responses were clearly demonstrated in each pig.

TABLE IV Potentiation by exogenous secretin of the pancreatic exocrine response to vagal stimulation

Number of expts.	Flow of juice %	Protein conc. %	Bicarbonate conc.	Protein output %	Bicarbonate output
13	184 (153-312)	48 (21-80)*	111 (103-126)	89 (79-201)	214 (164-315)

Submaximal or maximal vagal stimulation (5 min) was carried out 30 and 60 min after injection of secretin 0.5 C.U./kg. The data are presented as the response at 30 min in per cent of response to the stimulation at 60 min, with each animal being its own control, and expressed as the median followed by the 95% confidence limits. Because the basal rate of secretion (60 min after secretin) as well as the secretin-stimulated secretion (30 min after secretin, Table I) constituted less than 5% of the total response to vagal stimulation, these values have not been subtracted.

*Significant changes.

TABLE V Potentiation by acidification of the duodenal bulb of the pancreatic exocrine response to stimulation.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
infusion at pH 4					
0-10 min	0.15 (0-0.48)	—	—	—	—
10-20 min	0.03 (0-0.06)	—	—	—	—
4 Hz Vagal stimulation					
20-25 min	18.1 (13.2-30.0)	55 (36-184)	108 (70-122)	1.17 (0.5-4.24)	1.90 (1.35-3.64)
infusion at pH 1					
0-10 min	0.51 (0-1.2)	—	—	—	—
10-20 min	0.5 (0.6-4.0)*	—	—	—	—
4 Hz Vagal stimulation					
20-25 min	42 (25.2-57.5)	28 (24-54)	125 (85-145)	1.18 (0.63-3.10)	3.56 (2.47-8.26)
Response to vagal stimulation at pH 1 as per cent of the response at pH 4	225 (192-467)	61 (16-88)*	127 (89-140)*	149 (39-371)*	281 (185-653)*

* The duodenal bulb was perfused with buffers of pH 4 and pH 1 for 25 min in 4 pigs. After 20 min vagal stimulation was performed for 5 min at 4 Hz. Results are presented as the median and the total range. * Significant changes.

d) Potentiation by secretin of the response to vagal stimulation

1) In 13 pigs submaximal or maximal vagal stimulations were carried out 30 min after secretin administration, and repeated again after another 30 min. Thirty min after the secretin injection, the plasma concentration of secretin was approximately twice as high as the basal concentration (see below) and the pancreatic flow of juice slightly increased (Table I). At 60 min both parameters had reached basal levels.

If the fluid and bicarbonate response to vagal stimulation was merely added to the secretory response to secretin 30 min after the injection, one would have expected a combined

TABLE VI The effect of splanchnicotomy on the pancreatic exocrine response to vagal stimulation.

	Flow of juice	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
Vagal stimulation 3-20 Hz and bilateral splanchnicotomy	5.				
Medians (range)	125 (79-369)	103 (75-124)	110 (88-170)	144 (84-381)	125 (70-409)

* Per cent of the response to vagal stimulation before splanchnicotomy with each animal being its own control

TABLE VII. Pancreatic exocrine response to electrical stimulation of the left splanchnic nerve.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Secretin 0.5 C.U./kg. Second 5 min period (5)*	31.6 (36-60)	0.4 (0.2-1.9)	140 (132-156)	0.02 (0.007-0.09)	7.0 (4.3-9.1)
Vagal stimulation at 8-20 Hz. First 5 min (6)	33.9 (22.2-48.0)	114 (85-167)	131 (1-4-138)	3.8 (2.2-6.9)	4.3 (0-9.4)
Splanchnic stimulation at 10-20 Hz (10)	0.30 (0.0-1.80)				

Pancreatic secretion during splanchnic stimulation was investigated in 10 pigs, in 8 of these stimulation was also performed, and 6 received secretin 0.5 C.U./kg. The data are presented as the med followed by the total range.

response which exceeded the response 60 min after secretin (flow 31 ml/h, Table II) by 2.1 ml/h (Table I) *i.e.* 7%. However we found fluid and bicarbonate responses which constituted 184 and 214% of the responses without secretin (Table IV), which indicates that slightly elevated secretin concentrations markedly potentiate the fluid and bicarbonate response to vagal stimulation. The protein concentration was reduced to 48%, but the output was unchanged (89%).

2) In 4 pigs the vagi were stimulated with low frequency (4 Hz) during simultaneous perfusion of the duodenal bulb with isotonic buffers of pH 4 and 1 respectively but similar titratable acidity. The perfusion of the duodenal bulb at pH 4 did not affect pancreatic secretion, while a stimulated flow of juice was noted in all pigs during perfusion at pH 1. The volume and bicarbonate response to vagal stimulation under these circumstances was again markedly potentiated as shown in Table V.

e) The splanchnic nerves

The results of vagal stimulation before and after sectioning of the splanchnic nerves are shown in Table VI. In 4 of 5 pigs flow, protein and bicarbonate output were enhanced after splanchnicotomy ($p=0.075$ U-test).

TABLE VIII. Pancreatic exocrine response to simultaneous splanchnic and vagal stimulation

	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Protein output %	Bicarbonate output %
Vagal stimulation, 8 Hz, splanchnic stimulation 10 Hz (n=5). Median (range)	7.6 (4-37.5)*	85.7 (67-137)	90 (42-98)	10.5 (3-49.7)	5.6 (4-33.8)

Per cent of the response to vagal stimulation alone with each animal being its own control.
Significant changes.

TABLE IX. Pancreatic exocrine response to prolonged vagal stimulation.

Interval of collection (min.)	Flow of juice	Protein conc.	Bicarbonate conc. "	Protein output	Bicarbonate output "
0-5	100	100	100	100	100
5-10	97 (70-111)	77 (47-127)	101 (100-110)	70 (35-127)	100 (71-111)
10-15	89 (50-106)	81 (50-121)	102 (93-110)	60 (47-103)	89 (49-116)
15-20	82 (73-100)	64 (45-104)	101 (74-106)	50 (34-84)	81 (74-89)
20-25	93 (64-113)	69 (35-103)	106 (97-121)	44 (32-96)	96 (68-119)
25-30	133 (56-153)	49 (37-74)	103 (94-126)	66 (23-97)	141 (53-166)

Vagal stimulation (8 Hz) was continued for 30 min in 6 pigs. Juice was collected in 5-min periods. The values represent per cent of the response during the first 5 min, with each pig being its own control (median and total range).

Splanchnic nerve stimulation was performed in 10 pigs, which showed similar responses to secretin and vagal stimulation as the others (Table VII). The pancreatic exocrine secretion did not increase above basal levels. It was, however, not possible to determine if splanchnic stimulation inhibited the basal secretion. The efficiency of the splanchnic stimulation was evidenced by a pronounced rise in arterial blood pressure a few seconds after the start of stimulation. In 5 pigs the response to spontaneous submaximal vagal stimulation (8 Hz) and splanchnic stimulation was examined. The results which appear in Table VIII are presented as the response to combined stimulation in per cent of the response in the same pig to vagal stimulation at the same frequency alone. The protein concentration remained unchanged but the other 4 parameters of secretion were inhibited, the greatest reduction being for flow of juice.

7) Reproducibility of responses

Table IX and X show the results of prolonged (30 min) and repeated vagal stimulation. It can be seen, that an increase in exocrine secretion lasted as long as stimulation was continued, and that similar responses were elicited by repeated stimulations.

8) Pharmacological analysis

Table XI shows the results of vagal stimulation at various frequencies after various doses of atropine. It is obvious that atropine does not inhibit the vagally stimulated flow of juice, the bicarbonate concentration or the bicarbonate output. The protein concentration how-

TABLE X. Pancreatic exocrine response to vagal stimulation. Reproducibility (8 expts. in 7 pigs).

	Flow of juice ^a	Protein conc.	Bicarbonate conc.	Protein output ^a	Bicarbonate output ^a
Median (range)	100 (58-120)	96 (70-147)	107 (96-129)	105 (81-115)	109 (73-155)

^a Per cent of response to first stimulation. The stimulations were repeated after 0.3-3 h. Median and total range.

TABLE VII Pancreatic exocrine response to electrical stimulation of the left splanchnic nerve.

	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
Secretin 0.5 C.U./kg. Second 5 min period (6) ^a	51.6 (36-60)	0.4 (0.2-1.9)	140 (132-156)	0.02 (0.007-0.09)	7.0 (4.9-9.6)
Vagal stimulation at 8-20 Hz. First 5 min (6)	33.9 (22.2-48.0)	114 (85-167)	131 (124-138)	3.8 (2.2-6.9)	4.3 (3.0-6.0)
Splanchnic stimulation at 10-20 Hz (10)	0.30 (0.0-1.80)				

Pancreatic secretion during splanchnic stimulation was investigated in 10 pigs, 11 of these a vagal stimulation was also performed, and 6 received secretin 0.5 C.U./kg. The data are presented as the median followed by the total range.

response which exceeded the response 60 min after secretin (flow 31 ml/h, Table II) by 2.1 ml/h (Table I) i.e. 7%. However we found fluid and bicarbonate responses which constituted 184 and 214% of the responses without secretin (Table IV), which indicates that slightly elevated secretin concentrations markedly potentiate the fluid and bicarbonate response to vagal stimulation. The protein concentration was reduced to 48%, but the output was unchanged (89%).

2) In 4 pigs the vagi were stimulated with low frequency (4 Hz) during simultaneous perfusion of the duodenal bulb with isotonic buffers of pH 4 and 1 respectively but similar titratable acidity. The perfusion of the duodenal bulb at pH 4 did not affect pancreatic secretion, while a stimulated flow of juice was noted in all pigs during perfusion at pH 1. The volume and bicarbonate response to vagal stimulation under these circumstances was again markedly potentiated as shown in Table V.

c) The splanchnic nerves

The results of vagal stimulation before and after sectioning of the splanchnic nerves are shown in Table VI. In 4 of 5 pigs flow, protein and bicarbonate output were enhanced after splanchnicotomy ($p=0.075$ U-test).

TABLE VIII Pancreatic exocrine response to simultaneous splanchnic and vagal stimulation.

	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Protein output %	Bicarbonate output
Vagal stimulation, 8 Hz, splanchnic stimulation 10 Hz (n=5). Median (range)	7.6 (4-37.3)	85.7 (67-137)	90 (42-98)	10.5 (3-49.7)	5.6 (4-33.8) ^a

Per cent of the response to vagal stimulation alone with each animal being its own control.
Significant changes.

TABLE XIII. Pancreatic exocrine secretion after acetylcholine infused intraspleneally for 5 min.

Dose of infusion	Flow of juice ml/h	Protein conc. g/l	Bicarbonate conc. mmol/l	Protein output g/h	Bicarbonate output mmol/h
0.10-0.015 mg/min	$\left\{ \begin{array}{l} 1.3 \\ 4.8 \\ 9.0 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 110 \\ 243 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 95 \\ 110 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 0.52 \\ 2.18 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 0.53 \\ 0.81 \end{array} \right.$
0.4-0.05 mg/min (5)	10.2 (2.4-12.9)	75 (41-148)	98 (80-120)	0.66 (0.10-1.81)	0.99 (0.36-1.18)
1.2-0.15 mg/min	$\left\{ \begin{array}{l} 8.6 \\ 3.6 \end{array} \right.$	$\left\{ \begin{array}{l} 137 \\ 51 \end{array} \right.$	$\left\{ \begin{array}{l} 110 \\ 143 \end{array} \right.$	$\left\{ \begin{array}{l} 1.18 \\ 0.18 \end{array} \right.$	$\left\{ \begin{array}{l} 0.95 \\ 0.52 \end{array} \right.$
4-0.5 mg/min (7)	11.4 (3.8-34.8)	134 (64-153)	96 (61-118)	1.43 (0.36-5.16)	0.98 (0.64-4.1)
mg/min	$\left\{ \begin{array}{l} 6.7 \\ 4.8 \\ 2.2 \end{array} \right.$	$\left\{ \begin{array}{l} 120 \\ 236 \\ - \end{array} \right.$	$\left\{ \begin{array}{l} 86 \\ 90 \\ - \end{array} \right.$	$\left\{ \begin{array}{l} 0.81 \\ 1.13 \\ - \end{array} \right.$	$\left\{ \begin{array}{l} 0.57 \\ 0.43 \\ - \end{array} \right.$
0 mg/min	$\left\{ \begin{array}{l} 4.8 \\ 12.0 \end{array} \right.$	$\left\{ \begin{array}{l} 152 \\ 222 \end{array} \right.$	$\left\{ \begin{array}{l} 95 \\ 104 \end{array} \right.$	$\left\{ \begin{array}{l} 0.63 \\ 2.66 \end{array} \right.$	$\left\{ \begin{array}{l} 0.45 \\ 1.25 \end{array} \right.$
100 mg/min	$\left\{ \begin{array}{l} 8.3 \\ 3.8 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 257 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 105 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 0.85 \end{array} \right.$	$\left\{ \begin{array}{l} - \\ 0.38 \end{array} \right.$

The data presented as individual values or median followed by total range.

juice after ACh ex. secretin 36.7% (median range 6.9-75) flow ACh ex. vagal stimulation 1.9.5 (0.0-75), protein concentration, ACh ex. vagus 180% (127-259) bicarbonate concentration, ACh ex. vagus 75% (64-87).

ix In six pigs acetylcholine was infused in doses of 0.1-0.5 mg/min after the administration of atropine, 0.5 mg/kg. In no case was the exocrine secretion stimulated above basal levels. xiv In four pigs ACh 0.5 mg/min was infused after the administration of hexamethonium. The results are shown in Table XIV. Hexamethonium stimulated rather than inhibited the exocrine response.

xi In four pigs the exocrine response to vagal stimulation was investigated after beta-adrenergic blockade. In each case beta-blockade was evidenced by a slight lowering of blood pressure and a decrease in pulse rate. The results are shown in Table XV. The flow and protein

TABLE XIV. Pancreatic exocrine response to intrasplenic acetylcholine after pharmacological blockade.

Dose of acetylcholine and blockade	Flow of juice ml/h	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
0.1-0.5 mg/min 6, atropine 0.5 mg/kg	0.90 (0.0-2.2)	—	—	—	—
0.5 mg/min Hexamethonium 10-30 mg/kg	4	170 (82-258) ^a	144 (103-163) ^a	97 (82-118) ^a	239 (103-375) ^a
				239 (103-375) ^a	169 (67-306) ^a

^a Per cent of the response to the same dose of acetylcholine without blockade, with each animal being its own control. The data are presented as the median and total range.

TABLE XI Pancreatic exocrine response to vagal stimulation after atropine

Frequency and dose of atropine	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Protein output ^a %	Bicarbonate output %
2 Hz, mg/kg (6)	113 (52-233)	10.4 (4.9-37)*	132 (62-143)	12.9 (3.6-79)	137 (32-334)
8 Hz, 2 mg/kg (6)	111 (68-288)	5.5 (1.8-11.3)	115 (102-132)	6.3 (1.4-21.8)*	123 (68-340)
20 Hz, 2 mg/kg (12)	92 (71-144)	15.6 (7.7-23.2)*	100 (95-111)	18.2 (12.6-39.6)*	89 (65-279)
2-20 Hz, 0.5-2 mg/kg (25)	104 (76-144)	10.0 (6.9-14.0)	111 (102-117)	12.3 (9.0-18.0)*	111 (77-152)

Per cent of response without atropine (Table III) with each animal being its own control. The data are expressed as the median followed by the total range (2 and 8 Hz) or median followed by 95% limits of confidence (20 and 2-20 Hz) of the median.

*Significant changes.

ever and hence the protein output was markedly decreased in all experiments. In two pigs atropine was administered in high doses as a primed infusion (0.5 mg/kg and 0.5 mg/kg h), but also with this technique it was impossible to inhibit the exocrine response to vagal stimulation. The results of an atropine dose-response study are shown in Table XII. The decrease in protein concentration and output was dependent on the dose of atropine, whereas the flow and bicarbonate secretion remained unaffected.

Hexamethonium, on the other hand, completely abolished the response to vagal stimulation. In eight animals which all responded satisfactorily to secretin (flow 51.8 (36.0-60.1) ml/h) and vagal stimulation (flow 30 (22.2-66.0) ml/h) there was no detectable deviation from basal secretion during vagal stimulation after hexamethonium blockade (flow 0.2 (0.0-0.6) ml/h).

We then studied the exocrine response to intraarterially infused acetylcholine. The results are summarized in Table XIII. The pancreatic exocrine response to acetylcholine was dose dependent.

A stimulated flow was noted with 10 µg/min while maximum response required 0.5 mg/min. Higher doses were less effective. The high doses (> 10 mg/min) severely depressed cardiorespiratory functions of the animals. The juice was characterized by its high protein concentration (134.1 g/l) and a variable concentration of bicarbonate. When compared to the responses to maximal secretin and vagal stimulation, with each animal being its own control, the following results of stimulation with 0.5 mg/min ACh were obtained: flow of

TABLE XII Pancreatic exocrine response to vagal stimulation after various doses of atropine

Frequency and dose of atropine	Flow of juice %	Protein conc. %	Bicarbonate conc. %	Prot. in output ^a %	Bicarbonate output %
8 Hz	100	100	100	100	100
8 Hz + 0.005 mg/kg	150	59	120	89.1	180
8 Hz + 0.05 mg/kg	150	8.7	129	13.1	193
8 Hz + 0.5 mg/kg	133	4.9	132.0	6.3	176

Per cent of response without atropine. Stimulation for 5 min.

XVII. Effect of vagal stimulation on the secretin concentration in portal venous plasma during acidification of the duodenal bulb.

Minutes after start of stimulation

15 10 5 0 0.5 1 3 5 10 15

secretin conc. pmol/l mean and total range),	2.1 (0-6.4)	3.5 (1.7-7.6)	2.8 (0.1-13.5)	4.5 (1.4-8.4)	4.4 (1.4-7.4)	4.0 (1.4-7.0)	2.9 (1.4-6.0)	4.6 (1.3-7.7)	4.0 (1.6-9.6)	3.3 (2.0-10.3)
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stimulation was carried out at 4 Hz in 4 pigs during perfusion of the duodenal bulb with buffer of pH 4 and buffer of pH 1.0. The differences in the plasma concentration of secretin during pH 1 and pH 4 are given in the Table, with each animal being its own control. The duodenal bulb was perfused from 5 min after the start of stimulation.

secretin concentration in portal plasma during perfusion of the duodenal bulb with citrate buffer at pH 4 did not differ from the concentration in non-perfused animals, vagal stimulation did not change this concentration. Perfusion of the duodenal bulb with citrate buffer at pH 1 caused a significant increase in the portal secretin concentration (Table XVII). Again, vagal stimulation did not influence the concentration. The results are expressed as the difference between the concentrations of secretin obtained during perfusion at pH 4 and pH 1 with each animal being its own control.

Discussion

The results presented above A) confirm and extend earlier observations on the importance of the vagal innervation in the control of pancreatic exocrine secretion (Harper 1972, Hick 1970 b, Thomas 1967, Prishaw 1967, Sjödin 1977). B) provide evidence, that the sympathetic nervous system may modulate the secretory response to vagal stimulation, C) suggest that vagal potentiation of hormonally regulated secretion is of great physiological importance and D) raise the question of the mechanism involved in the secretory response to vagal stimulation.

In the dog and the cat (Hickson 1970 b, Brown *et al.* 1967, Lenninger and Ohlin 1971) vagal stimulation enhances enzyme output but causes only a sparse flow of juice. It was therefore of considerable interest, when Hickson reported that in the pig vagal stimulation, apart from increasing protein secretion, causes a profuse flow of juice with a high content of bicarbonate (Hickson 1963, 1970 b). Circumstantial evidence has been presented that other species, including man (Comlenc *et al.* 1964, Thomas 1967) may respond similarly. Our findings confirm the potency of vagal stimulation on the secretion of the porcine pancreas. The flow and bicarbonate responses amounted to 59 and 55% of the maximal secretory response to secretin. The response was clearly frequency dependent with a half-maximal effective frequency of 2-4 Hz and a maximal effective frequency of 12 Hz (Table III) in 3 of 4 experiments. An increased flow of juice was observed with frequencies below 1 Hz. This is in

TABLE XV Pancreatic exocrine response to vagal stimulation after adrenergic blockade

	Flow of juice	Protein conc.	Bicarbonate conc.	Protein output	Bicarbonate output
Vagal stimulation 8-12 Hz + Propranolol 1 mg/kg. n=4 Median (range)	125 (70-153)	163 (112-349)	89 (7-109)	194 (140-332)	111 (51-146)
Vagal stimulation 8-12 Hz + Propranolol as above + Phenoxyben- zamine 1 mg/kg. n=5 Median (range)	142 (64-315)	101 (63-248)	100 (72-105)	143 (63-401)	144 (64-131)

Per cent of response to vagal stimulation without blockade with each animal being its own control

response were increased ($p=0.17$) whereas the bicarbonate response was not changed significantly.

Finally in 5 pigs the response to vagal stimulation was investigated after a combined α and β -adrenergic blockade. In these experiments, all fluid losses were replaced with Macroderex 6% in saline (Pharmacia, Uppsala, Sweden) to avoid excessive hypotension. Again the response to vagal stimulation was enhanced, when compared to stimulation before blockade ($p=0.075$ (Table XV)).

Secretin determinations

Blood samples for secretin determination were taken during vagal stimulation in 25 experiments in 17 pigs (Table XVI). Although there was no significant change in the secretin concentration in portal plasma at any time, the median value decreased by 15% immediately after the start of vagal stimulation. The arterial concentration of secretin (measured in 16 pigs), remained unchanged during stimulation.

In 10 pigs blood samples for secretin determination were obtained 30 min after the administration of secretin 0.5 Clinical Units/kg. The median concentration was 5.3 (2.7-8.6 pmol/l) significantly and approximately 2 times greater than the basal levels.

TABLE XVI Effect of vagal stimulation on the secretin concentration in portal venous plasma.

	Minutes after start of stimulation									
	-15	10	5	0	0.5	1	3	5	10	15
Plasma secretin pmol/l										
25 expts. in 17 animals	2.7	2.1	2.0	2.6	2.2	2.4	2.6	2.6	2.6	2.5
Median + 95% confidence limits of the median	(1.6-4.0)	(1.3-3.1)	(1.5-2.4)	(1.7-3.0)	(1.8-3.3)	(1.5-2.8)	(1.8-2.9)	(2.4-3.4)	(1.8-3.4)	(1.8-3.3)

Frequency of stimulation was 8-20 Hz in 13 expts. and 4 Hz in the remaining 12. Since the stimulation did not significantly influence the plasma secretin concentration in either group, the groups were combined in the Table.

TABLE XVII. Effect of vagal stimulation on the secretin concentration in portal venous plasma during acidification of the duodenal bulb.

	Minutes after start of stimulation									
	15	10	5	0	0.5	1	3	5	10	15
Secretin conc. pmol/l	21	33	28	43	44	40	39	46	49	33
secretin and total range),	10-64	(17-76)	(21-115)	(14-84)	(14-74)	(14-78)	(14-60)	(13-77)	(14-96)	(20-103)

and stimulation was carried out at 4 Hz in 4 pigs during perfusion of the duodenal bulb with buffer of pH 4.0 and 1 buffer of pH 1.0. The differences in the plasma concentration of secretin during pH 1 and 1.4 are given in the Table, with each animal being its own control. The duodenal bulb was perfused from 20 to 5 min after the start of stimulation.

The concentration of secretin in portal plasma during perfusion of the duodenal bulb with citrate buffer at pH 4 did not differ from the concentration in non-perfused animals, and vagal stimulation did not change this concentration. Perfusion of the duodenal bulb with citrate buffer at pH 1 caused a significant increase in the portal secretin concentration (Table XVII). Again, vagal stimulation did not influence the concentration. The results are presented as the difference between the concentrations of secretin obtained during perfusion at pH 4 and pH 1 with each animal being its own control.

Discussion

The results presented above A) confirm and extend earlier observations on the importance of the vagal innervation in the control of pancreatic exocrine secretion (Harper 1972, Hicks 1970 b, Thomas 1967, Prashaw 1967, Sjodin 1977), B) provide evidence, that the sympathetic nervous system may modulate the secretory response to vagal stimulation, C) suggest that vagal potentiation of humorally regulated secretion is of great physiological importance and D) raise the question of the mechanism involved in the secretory response to vagal stimulation.

In the dog and the cat (Hickson 1970 b, Brown *et al.* 1967, Lemminger and Ohlin 1971) vagal stimulation enhances enzyme output but causes only a sparse flow of juice. It was therefore of considerable interest, when Hickson reported that in the pig vagal stimulation, apart from increasing protein secretion, causes a profuse flow of juice with a high content of bicarbonate (Hickson 1963, 1970 b). Circumstantial evidence has been presented that other species, including man (Comline *et al.* 1964, Thomas 1967) may respond similarly. Our findings confirm the potency of vagal stimulation on the secretion of the porcine pancreas. The flow and bicarbonate responses amounted to 39 and 55% of the maximal secretory response to secretin. The response was clearly frequency dependent with a half-maximal effective frequency of 2-4 Hz and a maximal effective frequency of 12 Hz (Table III) in 3 experiments. An increased flow of juice was observed with frequencies below 1 Hz. This is in

agreement with the frequency response pattern of other gastrointestinal functions it be controlled by parasympathetic innervation (Hillarp 1960). The response to vagal stimulation was highly reproducible (Table X), and a stimulated rate of secretion could be maintained as long as the electrical stimulation was continued (Table IX).

Compared to the measured basal flow of juice the response to maximal vagal stimulation represent a 55-fold increase. The basal flow is comparable to that obtained in the pancreas from pigs of similar size, when perfused with a completely synthetic medium (Lindkjaer Jensen *et al.* (in press)).

B

Splanchnic stimulation strongly inhibited the response to simultaneous vagal stimulation mostly through an inhibition of the flow of juice (Table VIII). Furthermore, the response to vagal stimulation was enhanced after cutting the splanchnic nerves. Likewise, after and/or β -adrenergic blockade most animals showed increased responses. Thus, while nothing can be deduced about the mechanism of the sympathetic inhibitory effect the results suggest that the sympathoadrenal system may significantly modulate the pancreatic exocrine secretion in response to vagal activity.

This finding also implies that attempts to elicit vagally induced fluid and bicarbonate secretion by means of central glucopenia (insulin and 2-deoxy-glucose) are likely to fail because of the simultaneous activation of the sympathetic nervous system.

Stimulatory effects of splanchnic stimulation (Thomas 1967) could not be demonstrated.

C

Our finding of potentiation by secretin of the response to vagal stimulation is in agreement with previous findings in pigs (Magee and White 1965; Hickson 1970 a) and cat (Brown *et al.* 1967). The recent development of sensitive and accurate radioimmunoassays for secretin (Schaffalitzky de Muckadell and Fahrenkrug 1977 c) enabled us to ascertain that this phenomenon was studied during elevations of the plasma level of secretin which were comparable to those seen in physiological circumstances. The levels attained (5.3 and 11 pmol/l respectively) were well within the range found during and after ingestion of a meal (Schaffalitzky de Muckadell and Fahrenkrug (in press); Fahrenkrug and Schaffalitzky de Muckadell 1977). The pancreatic fluid and bicarbonate responses to vagal stimulation under these circumstances amounted to 184 and 214% (exogenous secretin) and to 225 and 270% (duodenal acidification) of the responses to vagal stimulation in control experiments in the same animals without elevations of the plasma concentration of secretin. These results have important physiological implications. It is reasonable to believe that the duodenal bulb (which was the only part of the duodenum perfused in these studies) is rather constantly exposed to fluid of low pH (close to 1) in contrast to the rest of the duodenum, where only occasional "spikes" of pH lower than 4-5 are found in the fasting state as well as during the ingestion of a meal (Schaffalitzky de Muckadell and Fahrenkrug (in press)). Our results prove, that acidification of the bulb alone is sufficient to stimulate the secretion of secretin and pancreatic secretion of fluid and bicarbonate, but the response is small. But if one adds to this a moderate vagal activity a greatly increased pancreatic secretin

traces, which is sufficient to explain a considerable part of the pancreatic secretion believed to take place after a meal.

The pancreatic secretion of protein was found to be markedly enhanced by vagal stimulation in agreement with previous investigations (Harper 1972, Thomas 1967, Proshaw 1967). The response was strongly inhibited by low doses of atropine, completely abolished by hexamethonium, mimicked closely by acetylcholine, the effects of which were also abolished by atropine but unaffected by hexamethonium. It is therefore reasonable to believe, that the vagal stimulation of protein secretion follows the classical pathways of the parasympathetic nervous system, *i.e.* we stimulate preganglionic fibres, the activity of which is transmitted via cholinceptive nicotinic receptors in autonomic ganglia to postganglionic fibres, which through the release of acetylcholine near cholinceptive muscarinic receptors triggers the target organ to secrete. The stimulated secretion of fluid and bicarbonate behaves differently however the most important difference being the complete atropine-resistance of the response to vagal stimulation. Also these findings are in agreement with previous investigations (Hickson 1970 b, Brown *et al.* 1967, Lenniger and Othlin 1971). Atropine resistance of effects mediated by parasympathetic nerves is known to occur in many systems of the organism (Jones and Nickerson 1975, Ambache 1955). The phenomenon was discussed in detail by Ambache (1955), who offered four explanations to this effect. Firstly the theory of Dale and Gaddum (1930) which implied the existence of specialized neuro-effector junctions, which an essentially "muscarine-like" action of the acetylcholine released by nerve endings was locally protected from the effects of atropine (the "proximity theory"). We find it difficult to accept this concept of atropine-protected choline receptors since atropine effectively blocked the stimulatory action of intraarterially administered acetylcholine.

It is also difficult to understand that cholinergic receptors for fluid and bicarbonate secretion should differ so much in this respect from the receptors for protein secretion, which apparently are readily accessible for atropine.

Secondly atropine resistance, which is due to destruction of atropine in the animal, should be considered, since a genetically determined "atropine-esterase" (atropine acyl hydrolase) activity is known to occur in rabbits (Goldstein *et al.* 1969). The negative results of continuous-infusion experiments, and the fact that the pancreatic protein secretion was strongly inhibited by low doses of atropine refutes this possibility. Similarly acute tolerance or "tachyphylaxis" would be a highly conjectural solution to the problem, since we never saw any inhibition, regardless of the time, dose, or number of injections given. Thirdly an atropine resistant secondary formation of a pharmacological agent *e.g.* bradykinin, was considered. To imply such a mechanism, however is but to refer the problem to a higher level of complexity.

Finally atropine resistance may be due to non-cholinergic transmission.

The response is not dependent on the availability of or the secretion rate of secretin, since it could be elicited in pigs after surgical removal of all tissue known to produce secretin (Hickson 1970 b) and since the secretin concentration in portal venous plasma was unaffected by vagal stimulation. The portal vein was selected for sampling to ensure the highest

degree of sensitivity but the results of secretin determination in arterial plasma confirm that the splanchnic production of secretin was unchanged by vagal stimulation. The lowering of the portal vein concentration of secretin is probably a consequence of the stimulating effect on portal flow elicited by vagal stimulation (Fahrenkrug *et al.* 1978).

Other gastrointestinal hormones (gastrins, cholecystokinin) may be liberated during vagal stimulation, and may be responsible for part of the pancreatic exocrine response in an intact animal but these hormones could not explain the secretory response to vagal stimulation after removal of the stomach and the small intestine (Hickson 1970 b). Furthermore gastrins and cholecystokinin are known to stimulate strongly the pancreatic secretion of enzymes and are therefore less likely to participate in the secretory response to vagal stimulation in our experiments since the protein response was easily inhibited by atropine.

The abdominal vagus is known to contain adrenergic fibres (Liedberg *et al.* 1973). An adrenergic contribution to the vagally stimulated pancreatic secretion is less likely in view of the lack of inhibitory effect of adrenergic blockade.

The results therefore indicate that acetylcholine may not be the final transmitter of stimulation to the pancreatic secretion of fluid and bicarbonate. Instead we suggest that the transmitter function is exerted by Vasoactive Intestinal Polypeptide (VIP) (Said 1975). VIP, a 28 amino acid peptide with a close similarity to secretin in structure and biological effects (Said 1975 Bodanszky *et al.* 1933) has been localized to neurons in many parts of the body (Larsson *et al.* 1976 a, b, Said and Rosenberg 1976, Bryant *et al.* 1976, Sundk *et al.* 1977, Larsson *et al.* 1977 a, b) and VIP-containing nerves have been shown to innervate acini and ganglia of the porcine pancreas (Larsson *et al.* 1978). By combined immunohistochemistry, immunocytochemistry and electron microscopy VIP has been localized to synaptic vesicles of VIP-containing nerves (Larsson 1977, Giachetti *et al.* 1977, Emerson *et al.* 1978). A release of VIP from nervous tissue has been elicited by the aid of potassium (Giachetti *et al.* 1977, Emerson *et al.* 1978). Furthermore, electrical stimulation of the vagus produces an atropine resistant splanchnic release of VIP in anesthetized pigs (Fahrenkrug *et al.* 1978, Schaffalitzky de Muckadell *et al.* 1977 b) and during stimulation an increased release of VIP from the pancreas can be demonstrated (unpublished observations).

We admit that the only reason to imply that acetylcholine is not the transmitter of the vagal effects on the pancreas is the fact that the fluid and bicarbonate secretion following vagal stimulation is atropine resistant. To imply that VIP is the transmitter we will have to explain in addition the effect of acetylcholine on the fluid and bicarbonate secretion (Table XIII) which is observable also in the totally isolated pancreas, perfused with a synthetic medium at constant flow (Holst, Lindner, Schaffalitzky de Muckadell and Fahrenkrug unpublished). Two explanations present themselves: either the acinar cells are equipped with muscarinic receptors, which are not innervated, and therefore do not participate in the vagally induced secretion, or the VIP-nerves may contain such receptors. That isolated acinar cells possess muscarinic receptors has been amply documented (Christophe *et al.* 1976) and the stimulated release of VIP after acetylcholine (Fahrenkrug *et al.* 1978) supports the idea of muscarinic receptors on the VIP nerves. A combination of both might well be the real explanation.

The development of sensitive and accurate radioimmunoassays for secretin has made

enable a delineation of the contribution of secretin in the control of pancreatic secretion of acid and bicarbonate. It appears that there is ample space for other control systems as well (Linos *et al.* 1977). Among these, the peptidergic nervous systems may very well turn out to be of primary importance.

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References

- DEBARGE, N., The use and limitations of atropine for pharmacological studies on autonomic effectors. *Pharmacol. Rev.* 1955, 7, 447-494.
- ODANICKY, M., Y. S. KATZNER and B. I. SAID, Biological activities of synthetic peptides corresponding to fragments of and to the entire sequences of the vasoactive intestinal polypeptide. *Proc. nat. Acad. Sci. (Wash.)* 1973, 70, 342-344.
- ROSEN, J. C. A. A. HARTER and T. SCHATZBERG, Penetration of secretin stimulation of the pancreas. *J. Physiol. (Lond.)* 1967, 190, 519-530.
- SEY, M. G., S. R. BLOOM, J. M. POLAK, R. H. ALBUQUERQUE, L. MOKLIN and A. O. E. PLAZER, Possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter substance. *Lancet* 1976, i, 991-993.
- SHOSTOVA, J. P. E. K. FRANDSEN, T. P. CONLON, G. KISHITA and J. D. GARDNER, Action of cholecystokinin, Cholecystic extract, and A 23187 on accumulation of Guanosine 3',5'-cyclic Monophosphate in dispersed guinea pig pancreatic acinar cells. *J. Biol. Chem.* 1976, 251, 4640-4645.
- OSLINE, R. S. J. C. D. HICKSON and M. A. MENAGE, Nervous fibres in the pancreas of different species. *J. Physiol. (Lond.)* 1964, 170, 47-48P.
- DALE, H. H. and J. H. GADSDON, Reactions of denervated voluntary muscle and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol. (Lond.)* 1930, 70, 109-144.
- DAVIS, W. M. and J. R. HEDGECOCK, The effect of vagal stimulation on plasma insulin and glucose levels in the baboon. *J. Physiol. (Lond.)* 1967, 192, 317-322.
- JÖNSSON, K. and C. LINTH, *Wissenschaftliche Tabellen*. J. R. Geigy A.G. Basel 1969, 105.
- SHOEN, P. C. J. FARBERGHEU, B. R. SCHAFFALITZKY DE MUCKADELL, T. M. JENSEN and L. L. IVERSEN, Vasoactive intestinal polypeptide (VIP): Vascular localization and potassium evoked release from rat hypothalamus. *Brain Res.* 1978, 143, 174-178.
- ANDERSSON, J. and O. B. SCHAFFALITZKY DE MUCKADELL, Plasma secretin concentration in man. Effect of intraduodenal glucose, fat, ascorbic acid, ethanol, HCl or ingestion of meal. *Eur. J. clin. Invest.* 1977, 7, 201-203.
- ANDERSSON, J., H. GALBO, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL, Influence of the endogenous nervous system on the release of Vasoactive Intestinal Polypeptide (VIP) from the porcine gastrointestinal tract. *J. Physiol. (Lond.)* 1978, 280, 405-422.
- MACBETH, A. S. I. SAID, R. C. RITAGLON and F. C. KOPPEL, Vasoactive intestinal polypeptide in brain: Localization in and release from isolated nerve terminals. *Proc. nat. Acad. Sci. (Wash.)* 1977, 74, 3434-3438.
- COULSTON, A. I. ASHONOW and S. M. KALMAN, *Principles of drug action*. Hoeber Medical Division, Harper and Row New York, 1969, 466-467 and 577-578.
- WILLIAMS, W. H., S. R. BLOOM, W. MITCHELL, W. DOMSCHKE, S. DOMSCHKE, D. BELONAVIK, L. DREILING and E. WITTMAN, Plasma secretin and pancreatic bicarbonate response to exogenous secretin in man. *Gut* 1977, 18, 191-193.
- HARTER, A. A., The control of pancreatic secretion. *Gut* 1972, 13, 308-317.
- HICKSON, J. C. D., The effect of stimulation of the vagus nerves on pancreatic secretion in the pig. *J. Physiol. (Lond.)* 1963, 144, 34-35P.
- HICKSON, J. C. D., The secretory and vascular response to nervous and hormonal stimulation in the pancreas of the pig. *J. Physiol. (Lond.)* 1970, 226, 299-322.

- HICKSON, J. C. D. The secretion of pancreatic juice in response to stimulation of the vagus nerves. *plg. J. Physiol. (Lond.)* 1970 b. 206: 257-297.
- HILLARP, N. Å., Peripheral autonomic mechanisms. I: *Handbook of Physiology* Am. Physiol. Soc., 1 Vol. II Washington D.C. 1960: 979-1006.
- INNES, I. R. and M. NICKERSON Atropine, Scopolamine and related antimuscarinic drugs. In Gootz, L. S. and Gilman A. (eds.) *The pharmacological basis of therapeutics*. Macmillan Publishing Co. New York. 1975: 514-532.
- ISENBERG, J. I., R. CANO and S. R. BLOOM, Effect of graded amounts of acid instilled into the duodenum on pancreatic bicarbonate secretion and plasma secretin in duodenal ulcer patients and normal subjects. *Gastroenterology* 1977 72: 6-8.
- LARSSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* 1977 173: 173-176.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL, Occurrence of nerves containing vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Lif. Sci.* 1977 a. 21: 2011-2014.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL, Vasoactive intestinal polypeptide occurs in the nerves of the female genital urinary tract. *Science* 1977 b. 197: 1374-1375.
- LARSSON, L. I., J. FAHRENKRUG, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL, Immunoreactive nerves in the pancreas by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Lif. Sci.* 1977 22: 773-780.
- LAUSON, L. I., J. FAHRENKRUG, O. B. SCHAFFALITZKY DE MUCKADELL, F. SUNDLER, R. H. VAN NISSEN and J. F. REHFELD Localization of vasoactive intestinal polypeptide to central and peripheral nerves. *Proc. Nat. Acad. Sci. (Wash.)* 1976 b. 73: 3197-3200.
- LARSSON, L. I., L. EDVINGSSON, J. FAHRENKRUG, R. HÅKANSSON, C. ÖRTENGREN, B. SCHAFFALITZKY DE MUCKADELL and F. SUNDLER, Immunohistochemical localization of a vasoactive polypeptide in the cerebrovasculature. *Brain Res.* 1976 a. 113: 400-404.
- LENNINGER, S. and P. OHLIN, The flow of juice from the pancreatic gland of the cat in response to electrical stimulation. *J. Physiol. (Lond.)* 1971 216: 303-318.
- LIEDBERG, G., K. C. NIELSEN, C. H. OWMAN and N.-O. SÖDERBERG, Adrenergic contribution to the abdominal vagus nerves in the cat. *Scand. J. Gastroenterol.* 1973 8: 177-180.
- LINDKJER-JENSEN, S., J. FAHRENKRUG, J. J. HOLST, O. V. NIELSEN and O. B. SCHAFFALITZKY DE MUCKADELL, Secretory effects of vasoactive intestinal polypeptide on the isolated perfused porcine pancreas. *Am. J. Physiol.* 1977 in press.
- LINDKJER-JENSEN, S., J. FAHRENKRUG, J. J. HOLST, C. KÜHL, O. V. NIELSEN, O. B. SCHAFFALITZKY DE MUCKADELL and T. SCHWARTZ, Secretory effects of secretin on the isolated perfused, porcine pancreas. *Amer. J. Physiol.* 1977 in press.
- LLANOS, O. L., S. J. KONTUREK, P. L. RAYFORD and J. C. THOMPSON, Pancreatic bicarbonate secretion and secretin responses to meals varying in pH. *Amer. J. Physiol.* 1977 233: E41-E46.
- MAGEE, D. F. and T. T. WHITE, Influence of vagal stimulation on secretion of pancreatic juice in the dog. *Ann. Surg.* 1965 161: 605-607.
- PRIESTLY, R. M. Integration of nervous and hormonal mechanisms of external pancreatic secretion. In *Handbook of Physiology* (ed. C. F. Code), sect. 6, vol. II American Physiological Soc. Washington D.C. 1967: 997-1005.
- RHODES, R. A., H. H. T. and W. Y. CHEY, Observations on plasma secretin levels by radioimmunoassay in response to duodenal acidification and to meat meal in humans. *Amer. J. Dig. Dis.* 1976 21: 871-874.
- SAID, S. I. Vasoactive intestinal polypeptide (VIP): Current status. In Thompson, J. C. (ed.) *Gastrointestinal hormones*. Austin, Texas: University of Texas Press. 1975: 591-597.
- SAID, S. I. and R. N. ROSENBERG, Vasoactive intestinal polypeptide: Abundant immunoreactivity in cell lines and normal tissue. *Science* 1976 192: 907-908.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG, Radioimmunoassay of secretin in plasma. *Scand. J. Clin. Lab. Invest.* 1977 c. 37: 155-162.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG, Secretion pattern of secretin in man. Regulation by gastric acid. *Gut* in press.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST, Plasma secretin concentration and pancreatic exocrine secretion after intravenous secretin and duodenal HCl. *Scand. J. Gastroenterol.* 1977 a. 12: 67-72.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST, Release of vasoactive intestinal polypeptide (VIP) by electrical stimulation of the vagal nerves. *Gastroenterology* 1977 b. 72: 371-375.

- SCHAFALITZKY DE MUCKADELL, H. B., J. FAHRENERG, S. W. THORBERG and H. WERNING, Pancreatic response and plasma secretin concentration during infusion of low dose secretin in man. *Scand. J. Gastroenterol.* 1978 13 305-311.
- WILCOX, S. *Nonparametric statistics for the behavioral sciences*. McGraw Hill Kogakusha, Ltd., Tokyo 1965 936.
- WILSON, L., Electrical stimulation of nerves and secretion from digestive glands. In: Brooks, F. P. and Fry, P. W. Eds: *Nerves and the gut*. Charles R. Slack, Thorofare NJ 1977 1-13.
- WINDLER, F. J., ALLMETS, R., HÄKANSSON, S., INOUEMARSSON, J., FAHRENERG and O. B. SCHAFALITZKY DE MUCKADELL, VIP innervation of the gall bladder. *Gastroenterology* 1977 72, 1375-1378.
- SONMAI, J. Earl, Neural regulation of pancreatic secretion. In *Handbook of Physiology* Ed. C. F. Code, Sect. 6. of IL American Physiological Society Washington D.C. 1967 935-948.

- HICKSON, J. C. II. The secretion of pancreatic juice in response to stimulation of the vagus nerve. *dig. J. Physiol. (Lond.)* 1970 b 206: 257-297.
- HILLARP, N. Å. Peripheral autonomic mechanisms. In *Handbook of Physiology* Am. Physiol. Soc. 1 Vol. II Washington D.C. 1960: 979-1006.
- INNES, I. R. and M. NICKERSON. Atropine, Scopolamine and related antimuscarinic drugs. In Good L. S. and Gilman A. (eds.) *The pharmacological basis of therapeutics*. Macmillan Publishing Co. New York 1975: 514-532.
- ISENBERG, J. I. R. CANO and S. R. BLOOM. Effect of graded amounts of acid instilled into the duodenum on pancreatic bicarbonate secretion and plasma secretin in duodenal ulcer patients and normal subjects. *Gastroenterology* 1977 72: 6-8.
- LARSSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* 1977 173: 173-176.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL. Occurrence of vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci.* 1977 a 21: 981.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL. Vasoactive intestinal polypeptide occurs in the nerves of the female genitourinary tract. *Science* 1977 b 197: 1374-1375.
- LARSSON, L. I., J. FAHRENKRUG, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL. Innervation of the prostates by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci.* 1977 773: 773-780.
- LARSSON, L. I., J. FAHRENKRUG, O. B. SCHAFFALITZKY DE MUCKADELL, F. SUNDLER, R. HÅKANSSON, J. F. REHFELD. Localization of vasoactive intestinal polypeptide to central and peripheral nervous system. *Proc. natl. Acad. Sci. (Wash.)* 1976 b 73: 3197-3200.
- LARSSON, L. I., L. EDVINGSSON, J. FAHRENKRUG, R. HÅKANSSON, C. ÖRTENGREN, O. B. SCHAFFALITZKY DE MUCKADELL and F. SUNDLER. Immunohistochemical localization of vasoactive intestinal polypeptide in the cerebrovascular system. *Brain Res.* 1976 a 113: 400-404.
- LENNITZER, S. and P. OHLIN. The flow of juice from the pancreatic gland of the cat in response to stimulation. *J. Physiol. (Lond.)* 1971 216: 303-318.
- LIEDBERG, G., K. C. NIELSEN, C. H. ÖRTENGREN and H.-O. SÖDERBERG. Adrenergic contribution to the abdominal vagus nerves in the cat. *Scand. J. Gastroenterol.* 1973 8: 177-180.
- LINDKJÆR-JENSEN, S., J. FAHRENKRUG, J. J. HOLST, O. V. NIELSEN and O. B. SCHAFFALITZKY DE MUCKADELL. Secretory effects of vasoactive intestinal polypeptide on the isolated perfused porcine pancreas. *J. Physiol.* 1977 in press.
- LINDKJÆR-JENSEN, S., J. FAHRENKRUG, J. J. HOLST, C. KÖHL, O. V. NIELSEN, O. B. SCHAFFALITZKY DE MUCKADELL and T. SCHWARTZ. Secretory effects of secretin on the isolated perfused porcine pancreas. *Amer. J. Physiol.* 1977 in press.
- LLANOS, O. L., J. KONTUREK, P. L. RAYFORD and J. C. THOMPSON. Pancreatic bicarbonate, gastrin, and secretin responses to meals vary with pH. *Amer. J. Physiol.* 1977 233: E41-E46.
- MORLEY, D. F. and T. T. WHITE. Influence of vagal stimulation on secretion of pancreatic juice in the rat. *Surg.* 1965 161: 605-607.
- PRIESTLY, R. M. Integration of nervous and hormonal mechanisms for external pancreatic secretion. *Handbook of Physiology* (ed. C. F. Code), sect. 6, vol. II American Physiological Soc. Washington 1967: 997-1005.
- RHOODES, R. A., H. H. TAI and W. Y. CHEY. Observations on plasma secretin levels by radioimmunoassay in response to duodenal acidification and to a meat meal in humans. *Amer. J. dig. Dis.* 1976 21: 871.
- SATO, S. I. Vasoactive intestinal polypeptide (VIP). Current status. In Thompson, J. C. Ed. *Gastrointestinal hormones*. Austin Texas: University of Texas Press. 1975: 591-597.
- SATO, S. I. and R. N. ROSENBERG. Vasoactive intestinal polypeptide. Abundant immunoreactivity in the cells and normal tissue. *Science* 1976 192: 907-908.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG. Radioimmunoassay of secretin in plasma. *Scand. J. Clin. Lab. Invest.* 1977 c 37: 155-162.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG. Secretin pattern of secretin in man. Regulation by gastric acid. *Gut* 1977 in press.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST. Plasma secretin concentration and pancreatic exocrine secretion after intravenous secretin or intraduodenal HCl in anesthetized dogs. *Scand. J. Gastroenterol.* 1977 a 12: 267-272.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST. Release of vasoactive intestinal polypeptide (VIP) by electrical stimulation of the vagal nerves. *Gastroenterology* 1977 b 72: 373-377.

- SAFFALITZKY DE MUCKADELL, O. B. J. FAHRENERUD, S. W. THOMPSON and H. WERNER, Pancreatic response and plasma secretin concentration during infusion of low dose secretin in man. *Scand. J. Gastroenterol.* 1978. 11, 305-311.
- DEL, S. *Nonparametric statistics for the behavioral sciences*. McGraw-Hill Kogakusha, Ltd., Tokyo 1956.
- JOHN, L., Electrical stimulation of nerves and secretion from digestive glands. In: Brooks, F. P. and others, P. W. Eds: *Nerves and the gut*. Charles B. Slack, Thorofore N.J. 1977 1-13.
- ANDER, F. J. ALDRETT, R. HILKINSON, S. INGMANSON, J. FAHRENERUD and O. B. SCHAFFALITZKY DE MUCKADELL, VIP innervation of the gall bladder. *Gastroenterology* 1977 72, 1375-1378.
- OMAS, L. EARL, Neural regulation of pancreatic secretion. In: *Handbook of Physiology* Ed. C. F. Code, Sect. 6 vol. II. American Physiological Society Washington D.C. 1967 955-968.

- HICKSON, J. C. D. The secretion of pancreatic juice in response to stimulation of the vagus nerve. *plg. J. Physiol. (Lond.)* 1970 b. 706: 257-297.
- HOLLAR, N. A., Peripheral autonomic mechanisms. In *Handbook of Physiology*. Am. Physiol. Soc., 1 Vol. II Washington D.C. 1960: 979-1006.
- INTY, I. B. and M. NICKERSON, Atropine, Scopolamine and related antimuscarinic drugs. In Goss L. S. and Gilman A. (eds.), *The pharmacological basis of therapeutics*. Macmillan Publishing Co. New York, 1975: 514-532.
- ISENBERG, J. I., CANO and S. R. BLOOM, Effect of graded amounts of acid instilled into the duodenum on pancreatic bicarbonate secretion and plasma secretin in duodenal ulcer patients and normal subjects. *Gastroenterology* 1977 72: 6-8.
- LARSSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* 1977 173: 176.
- LARSSON, L. I., J. FAHREKRUG and O. B. SCHAFFALITZKY DE MUCKADELL, Occurrence of neuroactive vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci.* 1977 a. 21: 901.
- LARSSON, L. I., J. FAHREKRUG and O. B. SCHAFFALITZKY DE MUCKADELL, Vasoactive intestinal polypeptide occurs in the nerves of the female genitourinary tract. *Science* 1977 b. 197: 1374-1375.
- LARSSON, L. I., J. FAHREKRUG, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL, Immunohistochemical studies of vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci.* 1977 773-780.
- LARSSON, L. I., J. FAHREKRUG, O. B. SCHAFFALITZKY DE MUCKADELL, F. SUNDLER, R. HILL and J. F. REHFELD, Localization of vasoactive intestinal polypeptide to central and peripheral somata. *Proc. nat. Acad. Sci. (Wash.)* 1976 b. 73: 3197-3200.
- LARSSON, L. I., L. EDVINGSON, J. FAHREKRUG, R. HÅKANSSON, C. ÖRTENGREN, O. B. SCHAFFALITZKY DE MUCKADELL and F. SUNDLER, Immunohistochemical localization of a modulatory polypeptide (VIP) in cerebrovascular nerves. *Brain Res.* 1976 a. 113: 400-404.
- LENNINGER, S. and P. OHLIN, The flow of juice from the pancreatic gland of the cat in response to stimulation. *J. Physiol. (Lond.)* 1971 216: 303-318.
- LIEDBERG, G., K. C. NIELSEN, C. H. ÖRTENGREN and N.-O. SJÖBERG, Adrenergic contribution to the shivering response in the cat. *Scand. J. Gastroenterol.* 1973 8: 177-180.
- LINDKJER-JENSEN, S., J. FAHREKRUG, J. J. HOLST, O. V. NIELSEN and O. B. SCHAFFALITZKY DE MUCKADELL, Secretory effects of vasoactive intestinal polypeptide on the isolated perfused porcine pancreas. *J. Physiol.* in press.
- LIN KJER-JENSEN, S., J. FAHREKRUG, J. J. HOLST, C. KÜHL, O. V. NIELSEN, O. B. SCHAFFALITZKY DE MUCKADELL and T. SCHWARTZ, Secretory effects of secretin on the isolated perfused porcine pancreas. *Amer. J. Physiol.* in press.
- LLANG, O. L., S. J. KONTUREK, P. L. RAYFORD and J. C. THOMPSON, Pancreatic bicarbonate and gastrin responses to meals vary with pH. *Amer. J. Physiol.* 1977 233: E41-E46.
- MAGEE, D. F. and T. T. WHITE, Influence of vagal stimulation on secretion of pancreatic juice in the dog. *Surg.* 1965 161: 605-607.
- PRIEST, W. R. M., Integration of nervous and hormonal mechanisms for external pancreatic secretion. In *Handbook of Physiology* (ed. C. F. Code), sect. 6, vol. II American Physiological Soc. Washington D.C. 1967: 997-1005.
- RHODES, R. A., H. H. TAI and W. Y. CHIEH, Observations on plasma secretin levels by radioimmunoassay in response to duodenal acidification and to meat meal in humans. *Amer. J. Dig. Dis.* 1976 21: 111-114.
- SAID S. I., Vasoactive intestinal polypeptide (VIP): Current status. In Thompson, J. C. Ed. *Gastrointestinal hormones*. Aust. Texas. University Press. 1975: 591-597.
- SAID S. I. and R. N. ROSENBERG, Vasoactive intestinal polypeptide: Abundant immunoreactivity in cell lines and normal tissues. *Science* 1976 192: 907-908.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHREKRUG, Radioimmunoassay of secretin. *Scand. J. Lab. Invest.* 1977 37: 155-162.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHREKRUG, Secretion pattern of secretin in man. Regulation by gastric acid. *Gut* in press.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHREKRUG and J. J. HOLST, Plasma secretin concentration and pancreatic exocrine secretion after intravenous secretin or intraduodenal HCl. *Scand. J. Gastroenterol.* 1977 12: 267-272.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHREKRUG and J. J. HOLST, Release of vasoactive intestinal polypeptide (VIP) by electrical stimulation of the vagal nerves. *Gastroenterology* 1977 b. 72: 571-575.

- SHAFITALITZKY DE MUCKADELL, O. B., J. FARRERKRUU, S. W. TY BROSSEN and H. WERNING, Pancreatic response and plasma secretin concentration during infusion of low dose secretin in man. *Scand. J. Gastroenterol.* 1978. 13 305-311.
- SIL, S. *Nonparametric statistics for the behavioral sciences*. McGraw-Hill Kogakusha, Ltd Tokyo 1956.
- SPRY, L. Electrical stimulation of nerves and secretion from digestive glands. I. Brooks, F. P. and Vets, P. W. Eds. *Vivex and the gut*. Charles B. Slack, Thorofare N.J. 1977 1-13.
- STANDER, F. J. ALUMETS, R. HILKANKI, S. INOMAMATSU, J. FARRERKRUU and O. B. SHAFITALITZKY DE MUCKADELL, VIP innervation of the gall bladder. *Gastroenterology* 1977 72. 1375-1378.
- THOMAS, J., Earl, Neural regulation of pancreatic secretion. In *Handbook of Physiology* Ed. C. F. Code, p. 303-311. vol. II. American Physiological Society Washington D.C. 1967 935-968.

- HICKSON, J. C. D. The secretion of pancreatic juice in response to stimulation of the splanchnic pig. *J. Physiol. (Lond.)* 1970 b. 206: 257-297.
- HILLARP, N. Å. Peripheral autonomic mechanisms. I. *Handbook of Physiology*. Am. Physiol. Soc. I. Vol. II. Washington D.C. 1960: 979-1006.
- INNES, I. R. and M. NICKERSON. Atropine, Scopolamine and related antimuscarinic drugs. In: *Gast. L. S. and Gilman A. (eds.) The pharmacological basis of therapeutics*. Macmillan Publishing Co. New York. 1975: 514-532.
- ISENBERG, J. I., CANO and S. R. BLOOM. Effect of graded amount of acid instilled into the tank on pancreatic bicarbonate secretion and plasma secretin in duodenal ulcer patients and normal. *Gastroenterology* 1977. 72: 6-8.
- LARSSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* 1977. 73: 176.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL. Occurrence of vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci.* 1977 a. 21: 4.
- LARSSON, L. I., J. FAHRENKRUG and O. B. SCHAFFALITZKY DE MUCKADELL. Vasoactive intestinal peptide occurs in the nerves of the female genitourinary tract. *Science* 1977 b. 197: 1374-1375.
- LARSSON, L. I., J. FAHRENKRUG, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL. Localization of the pancreas by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci.* 1977. 21: 773-780.
- LARSSON, L. I., J. FAHRENKRUG, O. B. SCHAFFALITZKY DE MUCKADELL, F. SUNDLER, R. HÄLLÉN and J. F. REIFELD. Localization of vasoactive intestinal polypeptide in central and peripheral nerves. *Proc. nat. Acad. Sci. (Wash.)* 1976 b. 73: 3197-3200.
- LARSSON, L. I., L. EDVINGSSON, J. FAHRENKRUG, H. HÄLLÉN, C. ÖWMAN, O. B. SCHAFFALITZKY DE MUCKADELL and F. SUNDLER. Immunohistochemical localization of a vasodilatory polypeptide in cerebrovascular nerves. *Brain Res.* 1976 a. 113: 400-404.
- LENNINGER, S. and P. ÖHLIN. The flow of juice from the pancreatic gland of the cat in response to stimulation. *J. Physiol. (Lond.)* 1971. 218: 303-318.
- LIEDBERG, G., A. C. NIELSEN, C. H. ÖWMAN and M.-O. SJÖBERG. Adrenergic contribution to the splanchnic vagus nerves in the cat. *Scand. J. Gastroenterol.* 1973. 8: 177-180.
- LIND, A., JENSEN, B., J. FAHRENKRUG, J. J. HOLST, O. V. NIELSEN and O. B. SCHAFFALITZKY DE MUCKADELL. Secretory effects of vasoactive intestinal polypeptide on the isolated perfused porcine pancreas. *Am. J. Physiol.* in press.
- LINDER, JENSEN, S., J. FAHRENKRUG, J. J. HOLST, C. KÖHL, O. V. NIELSEN, O. B. SCHAFFALITZKY DE MUCKADELL and T. SCHWARTZ. Secretory effect of secretin on the isolated perfused porcine pancreas. *Amer. J. Physiol.* in press.
- LIANG, O. L., S. J. KONTUREK, P. L. RAYFORD and J. C. THOMPSON. Pancreatic bicarbonate, gastrin, and secretin responses to meals and gastric pH. *Amer. J. Physiol.* 1977. 233: E41-E46.
- MAGEE, D. F. and T. T. WHITE. Influence of vagal stimulation on secretion of pancreatic juice in the dog. *Ann. Surg.* 1965. 161: 605-607.
- PRIEDMAN, R. M. Integration of nervous and hormonal mechanisms for external pancreatic secretion. *Handbook of Physiology* (ed. C. F. Code), sect. 6, vol. II. American Physiological Soc. Washington D.C. 1967: 997-1005.
- RHOES, R. A., H. H. T. and W. Y. CHY. Observations on plasma secretin levels by radioimmunoassay in response to duodenal acidification and to a meal in humans. *Amer. J. Dig. Dis.* 1976. 21: 274-275.
- SAM, S. I. Vasoactive intestinal polypeptide (VIP): Current status. In: Thompson, J. C. Ed. *Gastrointestinal hormones*. Austin Texas. University of Texas Press. 1975: 591-597.
- SAM, S. I. and R. N. ROSENBERG. Vasoactive intestinal polypeptide: Abundant immunoreactivity in several cell lines and normal tissues. *Science* 1976. 192: 907-908.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG. Radioimmunoassay of secretin in plasma. *Scand. J. Lab. Invest.* 1977 c. 37: 155-162.
- SCHAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG. Secretion pattern of secretin in man: Regulation by gastric acid. *Gut* in press.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST. Plasma secretin concentration and pancreatic bicarbonate secretion after intravenous secretin or intraduodenal HCl in anaesthetized pig. *Scand. J. Gastroenterol.* 1977 a. 12: 267-272.
- SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENKRUG and J. J. HOLST. Release of vasoactive intestinal polypeptide (VIP) by electrical stimulation of the vagal nerves. *Gastroenterology* 1977 b. 72: 373-375.

12. SCHAFFALITZKY DE MUCKADELL, O. B., J. FAHRENGREN, S. WITT-BOOLSKY and H. WERNER, Pancreatic response and plasma secretin concentration during infusion of low doses secretin in man. *Scand. J. Gastroenterol.* 1978 13 305-312.
13. EL, S. *Nonparametric statistics for the behavioral sciences*. McGraw-Hill Kogakusha, Ltd., Tokyo 1966.
14. LIVES, L. Electrical stimulation of nerves and secretion from digestive glands. In Brooks, F. P. and Wirt, P. W. Eds. *Nerves and the gut*. Charles B. Slack, Thorofare N.J. 1977 1-13.
15. DIER, P., J. ALLINGET, R. HÅKANSON, E. INGEMANSSON, J. FAHRENGREN and O. B. SCHAFFALITZKY DE MUCKADELL, VIP innervation of the gall bladder. *Gastroenterology* 1977 72 1373-1378.
16. EARL, J. Earl, Neural regulation of pancreatic secretion. In. *Handbook of Physiology* Ed. C. F. Code. vol. 6. vol. II. American Physiological Society Washington D.C. 1967 955-968.

- HICKSON, J. C. D. The secretion of pancreatic juice in response to stimulation of the vagus nerve. *plg. J Physiol (Lond)* 1970 b 206 257-297.
- HILLARP, N. Å., Peripheral autonomic mechanisms. In *Handbook of Physiology* Am. Physiol Soc. Vol II Washington D.C. 1960. 979-1006.
- INNES, I. R. and M. NICKERSON Atropine, Scopolamine and related antimuscarinic drugs. In C. L. S. and Gilman A. (eds), *The pharmacological basis of therapeutics* Macmillan Publishing Co. New York 1975 514-532.
- ISNERG, J. I. R. CANG and S. R. BLOOM, Effect of graded amounts of acid fast lipid I to the duct on pancreatic bicarbonate secretion and plasma secretin in duodenal ulcer patients and normal subjects. *Gastroenterology* 1977 72 6-8.
- LARSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochemistry* 1977 73 176.
- LARSON, L. I. J. FAHRENKRUG and O. B. SCHIAFFALITZKY DE MUCKADELL, Occurrence of vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci* 1977 a 21 381.
- LARSON, L. I. J. FAHRENKRUG and O. B. SCHIAFFALITZKY DE MUCKADELL, Vasoactive intestinal peptide occurs in the nerves of the female genital urinary tract. *Science* 1977 b 197 1374-1375.
- LARSON, L. I. J. FAHRENKRUG, J. J. HOLST and O. B. SCHIAFFALITZKY DE MUCKADELL, Inhibits the pancreas by vasoactive intestinal polypeptide (VIP) immunoreactive nerves. *Life Sci* 1977 73 780.
- LARSON, L. I. J. FAHRENKRUG, O. B. SCHIAFFALITZKY DE MUCKADELL, F. SUNDLER, R. HILAVEN, J. F. REHFELD, Localization of vasoactive intestinal polypeptide to central and peripheral nerves. *Proc natl Acad Sci (Wash)* 1976 b 73 3197-3200.
- LARSON, L. I. L. EDVINGSON, J. FAHRENKRUG, R. HÅKANSON, C. ÖRMAN, O. B. SCHIAFFALITZKY DE MUCKADELL and F. SUNDLER, Immunohistochemical localization of a vasodilatory polypeptide in cerebrovascular nerves. *Brain Res* 1976 113 400-404.
- LENNINGER, S. and P. OHLIN, The flow of juice from the pancreatic gland of the cat in response to stimulation. *J Physiol (Lond)* 1971 216 303-318.
- LITZKE, O. K. C. NIELSEN, C. H. ÖRMAN and N.-O. SÖDERBERG, Adrenergic contribution to the stimulation of vagus nerves in the cat. *Scand J Gastroenterol* 1973 8 177-180.
- LINDKJÆR, JENSEN, S., J. FAHRENKRUG, J. J. HOLST, O. V. NIELSEN and O. B. SCHIAFFALITZKY DE MUCKADELL, Secretory effect of vasoactive intestinal polypeptide on the isolated perfused porcine pancreas. *J Physiol* In press.
- LINDKJÆR, JENSEN, S., J. FAHRENKRUG, J. J. HOLST, C. KÜHL, O. V. NIELSEN, O. B. SCHIAFFALITZKY DE MUCKADELL and T. SCHWARTZ, Secretory effect of secretin on the isolated perfused porcine pancreas. *Amer J Physiol* In press.
- LEAHY, O. L., S. J. KONTUREK, P. L. RAYFORD and J. C. THOMPSON, Pancreatic bicarbonate secretion, and secretin responses: meals vary with pH. *Amer J Physiol* 1977 233 E41-E46.
- MAGILL, D. F. and T. T. WHITE, Influence of vagal stimulation on secretion of pancreatic juice in the rat. *Surg* 1965 161 605-607.
- PRESTIAW, R. H. Integration of nervous and hormonal mechanisms of external pancreatic secretion. In *Handbook of Physiology* (ed C. F. Code), sect 6 vol II American Physiological Soc. Washington D.C. 1967 997-1005.
- RHODES, R. A., H. H. TAI and W. Y. CHEY, Observations on plasma secretin levels by radioimmunoassay in response to duodenal acidification and to meal stimuli in humans. *Amer J dig Dis* 1976 21 873-879.
- SATO, S. I. Vasoactive intestinal polypeptide (VIP): Current status. I. Thompson, J. C. Ed. *Gastrointestinal hormone*. Austin Texas: University of Texas Press. 1975 591-597.
- SATO, S. I. and R. N. ROSENBERG, Vasoactive intestinal polypeptide: Abundant immunoreactivity in several cell lines and normal tissues. *Science* 1976 192 907-908.
- SCHIAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG, Radioimmunoassay of secretin in plasma. *Scand J clin Lab Invest* 1977 c 37 155-162.
- SCHIAFFALITZKY DE MUCKADELL, O. B. and J. FAHRENKRUG, Secretin pattern of secretin in response to gastric acid. *Gut* In press.
- SCHIAFFALITZKY DE MUCKADELL, O. B. J. FAHRENKRUG and J. J. HOLST, Plasma secretin concentration and pancreatic exocrine secretion after intravenous secretin or intraduodenal HCl in anesthetized pigs. *Scand J Gastrointest* 1977 a 12 267-272.
- SCHIAFFALITZKY DE MUCKADELL, O. B. J. FAHRENKRUG and J. J. HOLST, Release of vasoactive intestinal polypeptide (VIP) by electrical stimulation of the vagal nerves. *Gastroenterology* 1977 b 72 373-375.

Methods

Recordings from left and right sides of the CT were obtained in Sprague-Dawley rats weighing between 250 and 450 g. Before the recording the CT on one side was interrupted in the middle ear. This was generally done either by injecting 0.2-0.4 ml of 99.5% alcohol through the tympanic membrane under light Hypnorm[®] anaesthesia or by severing the CT in the middle ear. In some animals colchicine, 5 mg/ml, or Marcaine 5% long lasting local anesthetic, was injected at the same site. Marcaine was administered every 6 h as used. The animals were then left for time periods ranging from 1 h to 3 days. After this period the rats were anaesthetized with Methohexal and Hypnorm. The CT was dissected, cut and put on recording electrodes, the tongue was then stimulated with solutions of 0.3 or 0.1 M NaCl, 0.02 M epinephrine hydrochloride, 0.03 or 0.03 citric acid and 0.3 M sucrose. The stimuli were applied to the tongue for about 3 s, repeated. The water rises for about 30 s.

The nerve activity was recorded as automated trace and the average magnitude of 10 automated recordings to particular taste stimulus at each time interval was determined and used as measure of the taste response.

In 5 rats the right facial nerve was cut between the geniculate ganglion and the CNS 3-4 weeks before recording experiment. This was done to cause the selective degeneration of the efferent fibres in the CT nerve. The technique has been described elsewhere (Parbajan and Hellkant 1973).

Results

Recordings from normal rats

Since it is possible, though unlikely that there is a systematic difference between the responses of the right and left CT the response of nerves from the two sides were compared in 12 rats. The result is shown in Fig. 1 in which the amplitudes of the responses of the right and left CT to all taste stimuli used have been plotted. The regression line for these points was calculated ($y = 0.82x + 1.7$) and drawn in Fig. 1. The correlation coefficient between the responses of left and right CT is 0.93. Fig. 1 may indicate a difference between the two sides. However, if there was a difference between right and left CT this was not significant. Further, as can be seen from Fig. 1 there was a rather large variation in amplitude of the responses from animal to animal. There was no difference in the shape of the responses from right and left CT.

Recordings from denervated rats after injection of alcohol

Fig. 2 shows data from 85 rats. The magnitude of the responses to 0.3 M NaCl has been plotted against the time after the CT was interrupted in the middle ear. Each animal was tested only at one time interval. It is evident that the magnitude of the response decreased with time after destruction.

After about 5 h, the response in the nerve had declined to about 50%. No response at all could be recorded after 15 h in any of the 12 animals studied at this time interval.

As a comparison the average response magnitude of the CT of the other side has been included—the horizontal line.

In addition to the decline in response with time there is an indication of a stage of increased responsiveness during the first hours after the nerve has been cut. There is a strong correlation between the response magnitude at each interval and postoperative time ($r = 0.86$) which is best described by the equation

$$y = 77 - 28 \ln x$$

Fig. 2 is based on data from animals in which injections of alcohol in the middle ear were used to interrupt the CT. Since it might be thought that diffusion of alcohol along the

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Decline and disappearance of taste response after interruption of the chorda tympani proper nerve of the rat

By

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Abstract

HILLEKANT G., V. GOPAL and Y. NINOMIYA. *Decline and disappearance of taste response after interruption of the rat chorda tympani proper nerve* Acta physiol. scand. 1979; 105: 52-57.

The response amplitude in the chorda tympani proper nerve of rats to taste stimulation has been studied at different times after interruption of the nerve in the middle ear. The results show that the response in the nerve declined and disappeared completely within 15 h after the nerve was interrupted. As a first sign of deteriorating function a loss of hyperaesthesia was observed. Results obtained during application of local anesthetic or colchicine and section at different distances from the tongue indicate that the decrease in response was the result of an interruption of axoplasmic flow from the nerve cell bodies in the geniculate ganglion to the taste buds.

The fact that the morphological integrity of gustatory structures depends on intact gustatory nerves is well documented (cf. Kennedy 1972). In the rat, studies have shown the disappearance of taste buds after section of gustatory sensory nerves such as the glossopharyngeal (Guth 1957) or chordal-lingual (Whiteside 1927) and regeneration of taste buds after innervation (Guth 1958, Oakley 1967, Zalewski 1969). Recent electron microscope studies of these structures have not only confirmed this but have also demonstrated the sequence of degenerative changes at the cellular level. Thus in the study by Farbman (1969) the first morphological changes in extra and intra-gemmal nerve endings were observed in 30 specimens as early as 3-6 h after the combined chorda-lingual nerve had been cut. More consistent degenerative changes in these structures were observed between 12 and 24 h. Such morphological changes may be reflected in changes of function of these structures, as assessed by electrical recordings during an acute experiment. This paper describes changes of function in the peripheral sense of taste after section of the chorda tympani proper nerve (CT) in rat.

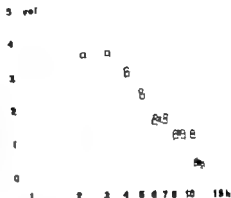


Fig. 3 The diagram shows the decline and disappearance of the CT response in one animal during repeated stimulation with 0.03 M citric acid (□) and 1 M NaCl (○). During the recording period the eye was kept as moist as possible. On four occasions the eye was removed and the nerve raised with Ringer solution (RL) which was then replaced with control.

though the response of the other side has disappeared. From this it can be concluded that the presence of anesthesia did not change the events described.

Recordings from denervated rats after injection of colchicine or local anesthetic

In addition to conducting impulses, gustatory nerves may transport substances which are vital for the maintenance of taste buds. Local anesthetic and colchicine can be used experimentally to differentiate between impulse propagation and axonal transport. The local anesthetic blocks impulses but leaves axoplasmic transport relatively unaffected (Byers *et al.* 1973), while colchicine allows the passage of impulses (Cuenod *et al.* 1974) but blocks the transport mechanism. Thus in 6 rats a long-acting local anesthetic was injected every sixth hour into the ear instead of alcohol. The results are illustrated by the squares in Fig. 4. No conclusions can be drawn. A response could be recorded for a much longer time than when alcohol was used. But the anesthetic did not leave the response unaffected which corroborates with the observations of Byers *et al.* (1973). Colchicine, on the other hand, caused a decline and disappearance in the same way as if the nerve had been interrupted by alcohol (not shown in Fig. 2). From this it seems that the mere interruption of impulse transmission was not the cause for the decline and disappearance of taste sensitivity described here.

In summary these results show that interruption of the CT in the middle ear makes the CT of rats insensitive to stimulation of the tongue with taste solutions within about 15 h. The interruption of the nerve may initially cause an increase in the response which then diminishes until no response can be recorded.

Discussion

The results of this study show that the response in the rat CT declines and disappears completely within 15 h after the nerve has been cut in the middle ear.

The observations described here were obtained in rats. There is no doubt that, though the general course is the same in other animals, the velocity of the decline differs. Thus in Rhesus monkeys we have recorded response to taste stimulation in the chorda tympani nerve for more than 24 h after cutting the CT. On the other hand, the decline is faster in the

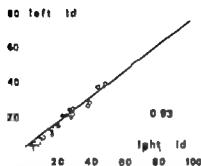


Fig. 1

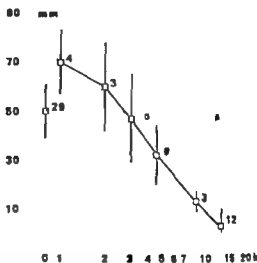


Fig. 2

Fig. 1. The amplitudes of the stimulated response of the right and left CT nerves on the same rat have been plotted against each other during stimulation with 0.3 M NaCl (\square), 0.02 M citric acid (\circ), 0.02 M quinolone hydrochloride (\circ) and 0.3 M sucrose (\blacksquare). Data from 12 rats were used.

Fig. 2. The magnitude of the responses in the CT of 85 rats to stimulation with 0.3 M NaCl was plotted against the time after the interruption of the CT in the middle ear. The diagram shows that the response decreased with time and disappeared within 15 h. The squares (\blacksquare) illustrate the response obtained after injection of a local anesthetic in the ear. The horizontal line illustrates the average magnitude of the CT of the other side. The vertical bars illustrate the SD of these values.

nerve to the recording site resulted in the effects illustrated in Fig. 2. In a few animals the nerve was cut where it passes near to the tympanic membrane. The results obtained did not differ from those described. Thus the decline was not caused by local effects of alcohol at the recording place.

The data of Fig. 2 were based on responses to the most effective stimulus, 0.3 M NaCl. The slope of decline of the responses to the other stimuli did not differ and as a rule we use 4 types of stimuli.

The possible effect of general anesthesia on the decline of response

The anesthetic is another factor which may affect the decline of the response described here. In an ordinary experiment the recording from the CT begins as soon as the nerve has been cut and the dissection is made under general anesthesia. It is possible that anesthesia may slow down the processes shown in Fig. 2. Therefore in 6 rats the CT nerves on one side were dissected and the taste response was recorded from the moment the nerve was cut until the response disappeared. Fig. 3 shows a recording from such an animal during stimulation with 0.1 M NaCl and 0.03 M citric acid. It shows the same feature as has been described: decline and disappearance of the response after about 12 h. The course of the decline of the individual nerve response was the same as those in Fig. 2. When the response in the CT had completely disappeared, the other side was dissected and a recording was made from the CT of that side. In 3 of these 6 animals mentioned above the nerves of this side had been interrupted with alcohol at the onset of the whole experiment. In these animals a response could be recorded. In the three other animals, whose CT nerves had not been touched earlier, there was no problem of recording a normal response to taste stimulation.

In summary we suggest that the decline and disappearance of the taste response after interruption of the CT was the result of an interruption of an axoplasmic flow in the gustatory fibres to the taste buds. The cessation of axoplasmic supply will probably affect the fine nerve endings first. As a first sign of deteriorating function a stage of hypersensitivity is a general feature.

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References

- BRIDGES, L. M. and R. L. SWALLMAN. Renewal of cells within taste buds. *J. cell. Biol.* 1965. 27 263-272.
- BRIDGES, D. W., J. B. CURT, M. A. HOWLEY, L. B. JONES, J. M. KALISZEWski, W. C. LAWLER and R. OASLEY. Commemorated by B. Oatley at the Vth International symposium of Olfaction and Taste, Paris, 1977.
- CHYER, M. R., R. R. FINE, R. D. KROVITZ, A. E. HINDENBURY and M. E. MANNING. Effects of Lidocaine on axonal morphology, microtubules and rapid transport in rabbit vagus nerve *in vitro*. *J. Neurobiol.* 1973. 4 125-143.
- LEWIS, M. P., M. MARKS, E. NEIDERER and K. AUSTY. Effects of degeneration and axoplasmic transport blockade on synaptic ultrastructure, function and protein composition. In *Dynamics of degeneration and growth in Neurosci.* Pergamon Press, 1974. 215-223.
- LEWIS, A. L. Fine structure of degenerating taste buds after denervation. *J. Embryol. exp. Morph.* 1969. 22 55-68.
- LEWIS, A. L. Differentiation of Taste Buds in Organ Culture. *J. cell. Biol.* 1972. 32 489-493.
- LEWIS, A. L. and G. HELLEKANT. Quantitative analyses of the fiber population, in rat chorda tympani proper nerves and fungiform papillae. *Amer. J. Anat.* In press.
- LYTH, L. Taste buds in the rat's circumvallate papilla after reinnervation by glossopharyngeal, vagus and hypoglossal nerve. *Anat. Rec.* 1958. 130 25-37.
- HELLEKANT, G. *Studies of the effect of ethyl alcohol on the receptors of the tongue*. Thesis Stockholm, 1963. 16 p.
- HELLEKANT, G. The effect of anesthetics on taste receptors. *Acta physiol. scand.* 1969. 76 361-368.
- HELLEKANT, G. The effect of changes in the blood flow to the tongue on the chorda tympani response in rat. *Acta physiol. scand.* 1971. 82 145-153.
- HELLEKANT, G. On the relation between the chorda tympani nerve response, arterial oxygen tension and blood flow in the tongue of the rat. *Acta physiol. scand.* 1971. 82 453-459.
- KANAKI, R. Y. and M. SAWCZUK. Influence of sensory neurons isolated from central nervous system on maintenance of taste buds and regeneration of barbs in the catfish *Ambloplites reticulatus*. *Amer. J. Physiol.* 1953. 174 146-148.
- KROVITZ, J. G. The effects of Transection of the Glossopharyngeal Nerve on the Taste Buds of the Circumvallate Papilla of the Rat. *Arch. oral Biol.* 1972. 17 1197-1207.
- JARLEY, B. Altered temperature and taste responses from cross-regenerated sensory nerves in the rat tongue. *J. Physiol. (Lond.)* 1967. 188 353-371.
- JARLEY, B. On the specification of taste neurons in the rat's tongue. *Brain Res.* 1974. 75 85-96.
- OLIVIERO-SALVADORI, C. Ultrastructural Modifications of Denervated Taste Buds. *Z. Zellforsch., Abt. Anat.* 1970. 108 397-414.
- TOOLEY, T. W. The relation of taste buds to their nerve fibres. *J. comp. Neurol.* 1934. 39 203-220.
- WERNER, J. H. Trophic influence of nerves on the development and maintenance of sensory receptors. *Amer. J. phys. Med.* 1974. 53 No 3. 127-142.
- WERNER, J. H. Nerve overlap in the gustatory apparatus of the rat. *J. comp. Neurol.* 1971. 44 363-377.
- ZALZWEIL, A. Changes in Phosphatase Enzymes following Denervation of the Vallate Papilla of the Rat. *Exp. Neurol.* 1968. 22 40-51.
- ZALZWEIL, A. Regeneration of Taste Buds after Reinnervation by Peripheral or Central Sensory Fibers of Vagal Ganglia. *Exp. Neurol.* 1969. 25 428-437.
- ZALZWEIL, A. Trophic influence of in vivo Transplanted Sensory Neurons on Taste Buds. *Exp. Neurol.* 1970. 29 442-447.
- ZALZWEIL, A. Regeneration of Taste Buds in Tongue Grafts after Reinnervation by Neurons in Transplanted Lumbosacral Sensory Ganglia. *Exp. Neurol.* 1973. 40 161-169.

gerbil glossopharyngeal nerve. Berland *et al.* 1977 reported that the response disappears within 1-4 h after the IXth nerve had been transected.

From the results with colchicine and Marcain it is evident that interruption of the flow of something in the nerve was the cause of the decline. Earlier studies (e.g. Guth 1952, Oakley 1974) as well as our own observations clearly demonstrate that after section of the taste nerves the taste buds of the tongue disappear. An uninterrupted connection to the taste buds is necessary for their maintenance and function (cf. Werner 1974). However, an uninterrupted connection with the brain is not necessary. Thus, we have recently shown (Farbman and Hellekant 1978) that as long as the connection between the neurons in the geniculate ganglion and the tongue remains, the structure and function of the taste bud will be maintained. Interruption of the axons between CNS and the ganglion will not cause their disappearance though of course it will abolish the ability of the animal to sense taste. Similar conclusions were drawn by Kamrin and Singer (1953) from data in catfish and by Zalewski in the rat (1969). The results do not allow conclusions on the type of substance that is transported but it seems evident from other studies (Zalewski 1968, 1969, 1970, Farbman 1972) that the cell bodies of the taste neurons synthesize a special type of substance either this synthesis occurs under the influence of some factor produced in the epithelium of the tongue and transported to the nerve cell bodies (Zalewski 1973) or is synthesized independent of the periphery.

The ultra structural changes accompanying the course of degeneration have been described by Farbman (1969). He observed the first changes within 3-6 h in some specimens. More consistent changes were observed at 12-24 h, which falls within the period of loss of function described here. It is implicit that the functional changes will precede the morphological. Therefore the difference in time between the morphological changes of Farbman and the functional changes described here may seem too small. However, Farbman cut the CT as it runs in the combined chorda-lingual nerve. This left a much shorter piece connected to the tongue than the method applied here and ought to cause a more rapid degeneration as previously claimed by Torrey (1934) for taste buds. Taking this factor into account, there is a close parallelism in time course between this study and that of Farbman (1969). From his study it is likely that the decrease in ability to respond described here could be due to changes in the nerve endings in which according to Farbman (1969) and Olivieri-Sanga-como (1970) the first degenerative changes were observed. Changes in the gustatory cell in the taste buds cannot, of course, be excluded as possible causes. However, it is unlikely that inability to mediate impulses in the CT was the cause, since the fibres of CT are capable of mediating impulses for a longer time.

The hypersensitivity observed during the first 1 or 2 h after the CT had been cut seems to be a physiological observation. A stage of hypersensitivity is in general the first step in a process leading to inexcitability. Similar effects can be observed initially when, for example, the blood supply to the tongue is occluded (Hellekant 1971a, b) or when chemicals such as alcohol or menthol which affect the membranes are applied to the tongue (Hellekant 1969). Bekler and Smallman (1965) reported also an initial enhancement of the response to stimulation with sucrose after colchicine, which may be considered to be in line with the observation.

In summary we suggest that the decline and disappearance of the taste response after interruption of the CT was the result of an interruption of an axoplasmic flow in the gustatory fibres to the taste buds. The cessation of axoplasmic supply will probably affect the nerve endings first. As a first sign of deteriorating function a stage of hypersensitivity is a general feature.

This study was supported by Karin and Alice Wallenbergs Stiftelse and Magnus Bergvalls Stiftelse.

References

- BRIDGES, L. M. and R. L. SMALLMAN, Regrowth of cells within taste buds. *J. cell. Biol.* 1965, 27, 263-272.
- ELLAND, D. W. J. S. CHOI, M. A. HENLEY, L. B. JONES, J. M. KALISZEWski, W. C. LAWLER and B. OAKLEY. Communication by B. Oakley at the Vth International Symposium of Olfaction and Taste, Paris, 1977.
- FRYER, M. R., B. R. FINE, R. D. KENNEDY, A. H. HENDERSON and M. E. MICHARD, Effects of Lidocaine on axonal morphology, microtubules and rapid transport in rabbit vagus nerve *in vitro*. *J. Neurobiol.* 1973, 4, 125-143.
- LINDO, M., P. MARZO, E. NEDERER and K. AKEBY. Effects of degeneration and axoplasmic transport blockade on synaptic ultrastructure, function and protein composition. In *Dynamics of degeneration and growth in Neurons*. Pergamon Press, 1974, 215-233.
- ARMAN, A. I. Fine structure of degenerating taste buds after denervation. *J. Embryol. exp. Morph.* 1969, 22, 35-48.
- ARMAN, A. I. Differentiation of Taste Buds in Organ Culture. *J. cell. Biol.* 1972, 52, 489-493.
- ARMAN, A. I. and G. HELLEKANT, Quantitative analysis of the fiber population, in rat chorda tympani proper nerves and fungiform papillae. *Ann. J. Anat.* 1973, in press.
- JUTH, L. Taste buds in the cat's circumvallate papillae after reinnervation by glossopharyngeal, vagus and hypoglossal nerves. *Ann. Rec.* 1958, 130, 25-37.
- HELLEKANT, G. *Studies of the effect of methyl alcohol on the receptors of the tongue*. Thesis Stockholm, 1965, 16 p.
- HELLEKANT, G. The effect of menthol on taste receptors. *Acta physiol. scand.* 1969, 75, 361-368.
- HELLEKANT, G. The effect of changes in the blood flow in the tongue on the chorda tympani response in the rat. *Acta physiol. scand.* 1971, 82, 145-153.
- HELLEKANT, G. On the relation between the chorda tympani nerve response, arterial oxygen tension and blood flow in the tongue of the rat. *Acta physiol. scand.* 1971, 82, 453-459.
- KAMURA, R. P. and M. BRONER, Influence of sensory neurons isolated from central nervous system on maintenance of taste buds and regeneration of buds in the catfish *Ameiurus nebulosus*. *Ann. J. Physiol.* 1953, 174, 144-148.
- LEVINE, J. H. The effects of transection of the Glossopharyngeal Nerve on the Taste Buds of the Catfish *Vallidus Papillae* of the Rat. *Arch. oral Biol.* 1972, 17, 1197-1207.
- OAKLEY, B. Altered temperature and taste responses from cross-regenerated sensory nerves in the rat's tongue. *J. Physiol. (Lond.)* 1967, 188, 353-371.
- OAKLEY, B. On the specification of taste neurons in the rat's tongue. *Brain Res.* 1974, 73, 85-96.
- SILVERSTEIN, C., Ultrastructural Modifications of Denervated Taste Buds. *Z. Zellforsch. Abt. Morphol.* 1970, 108, 397-414.
- TORNEY, T. W. The relation of taste buds to their nerve fibres. *J. comp. Neurol.* 1934, 39, 203-220.
- WERNER, J. K., Trophic influence of nerves on the development and maintenance of sensory receptors. *Ann. J. phys. Med.* 1974, 55, No. 3, 127-142.
- WHITSON, B. Nerve overlap in the gustatory apparatus of the rat. *J. comp. Neurol.* 1927, 44, 363-377.
- ZALZWEIL, A. Changes in Phosphatase Enzymes following Denervation of the Vallate Papillae of the Rat. *J. Exp. Neurol.* 1968, 22, 40-51.
- ZALZWEIL, A., Regeneration of Taste Buds after Reinnervation by Peripheral or Central Sensory Fibers of Vth Ganglion. *Exp. Neurol.* 1969, 25, 429-437.
- ZALZWEIL, A., Trophic influence of in vivo Transplanted Sensory Neurons on Taste Buds. *Exp. Neurol.* 1970, 29, 442-447.
- ZALZWEIL, A., Regeneration of Taste Buds in Tongue Grafts after Reinnervation by Neurons in Transplanted Lumbar Sensory Ganglia. *Exp. Neurol.* 1973, 40, 161-169.

gerbil glossopharyngeal nerve Berland *et al.* 1977 reported that the response declined within 1-4 h after the IXth nerve had been transected.

From the results with colchicine and Marcalin it is evident that interruption of the flow of something in the nerve was the cause of the decline. Earlier studies (e.g. Guth 1969, Oakley 1974) as well as our own observations clearly demonstrate that after section of taste nerves the taste buds of the tongue disappear. An uninterrupted connection to the taste buds is necessary for their maintenance and function (cf. Werner 1974). However, an uninterrupted connection with the brain is not necessary. Thus, we have recently shown (Farbman and Hellekant 1978) that as long as the connection between the neurons in the geniculate ganglion and the tongue remains, the structure and function of the taste bud will be maintained. Interruption of the axons between CNS and the ganglion will not cause their disappearance, though of course it will abolish the ability of the animal to sense taste. Similar conclusions were drawn by Kamrin and Singer (1953) from data in catfish and by Zalewski in the rat (1969). The results do not allow conclusions on the type of substance that is transported but it seems evident from other studies (Zalewski 1968, 1969, 1970; Farbman 1972) that the cell bodies of the taste neurons synthesize a special type of substance, either this synthesis occurs under the influence of some factor produced in the epithelium of the tongue and transported to the nerve cell bodies (Zalewski 1973) or is synthesized independent of the periphery.

The ultra structural changes accompanying the course of degeneration have been described by Farbman (1969). He observed the first changes within 3-6 h in some specimens. More consistent changes were observed at 12-24 h which falls within the period of loss of function described here. It is implicit that the functional changes will precede the morphological. Therefore the difference in time between the morphological changes of Farbman and the functional changes described here may seem too small. However, Farbman cut the CT where it runs in the combined chorda lingual nerve. This left a much shorter piece connected to the tongue than the method applied here and ought to cause a more rapid degeneration as previously claimed by Torrey (1934) for taste buds. Taking this factor into account, there is a close parallelism in time course between this study and that of Farbman (1969). From his study it is likely that the decrease in ability to respond described here could be due to changes in the nerve endings in which, according to Farbman (1969) and Olivieri-Sangalli *et al.* (1970), the first degenerative changes were observed. Changes in the gustatory cell in the taste buds cannot, of course, be excluded as possible causes. However, it is unlikely that inability to mediate impulses in the CT was the cause, since the fibres of CT are capable of mediating impulses for a longer time.

The hypersensitivity observed during the first 1 or 2 h after the CT had been cut seems to be a physiological observation. A stage of hypersensitivity is in general a first step in a process leading to inexcitability. Similar effects can be observed initially when, for example, the blood supply to the tongue is occluded (Hellekant 1971 a, b) or when chemicals such as alcohol or menthol which affect the membranes are applied to the tongue (Hellekant 1963, 1969). Bekler and Smallman (1965) reported also an initial enhancement of the response to stimulation with sucrose after colchicine, which may be considered to be in line with the observation.

Administration of adrenergic agonists and blockers. Isoprenaline (Isoprenalini sulphas, Pharm. Nord. 63) is given as an ether 1 or 20 min lasting intra-arterial infusion in doses from 0.01 to 1 $\mu\text{g/g}$ b.wt. Noradrenaline (noradrenalini bitartras) is given as an i.v. or i. infusion, lasting ether 1 or 20 min in doses 0.01 to 1 $\mu\text{g/g}$ b.wt. As it is found that the mice often died with signs of heart edema, neither ether infusion was used, the infusions were in most cases performed after pretreatment with 5 $\mu\text{g/g}$ dihydralazine, which causes hypotension without change of subcutaneous renin release (Bang and others 1976). The changes in renin release were neither influenced by this pretreatment, nor by the route of infusion (i. or v.) of noradrenaline, which in most cases is i.v. Some mice received the alpha-adrenergic blocker phenylephrine (Alfred Benzon) as one or repeated 1 min lasting infusions of 3 or 9 $\mu\text{g/g}$ b.wt.

Blood was drawn from femoral artery in capillary tubes moistened with 300 mM EDTA solution. Renin concentration was determined by the capture radioimmunoassay for angiotensin I (Pochet and Jorgensen 1974) and expressed in Goldblatt Units (G.U.) 10^{-6} ml $^{-1}$ by comparison with the Holly Hill hog renin standard, London.

Results

Effect of the β -adrenergic agonist Isoprenaline on release of renal and subcutaneous renin

The effect of isoprenaline on renal renin release in adrenalectomized mice is shown in Fig. 1 A and B. The maximum value of plasma renin concentration after a single infusion of 3 different doses 0.01, 0.1 and 1 $\mu\text{g/g}$ b.wt. is seen in Fig. 1 A, which shows a relation between dose and response. Fig. 1 B further shows that repeated infusions (indicated by

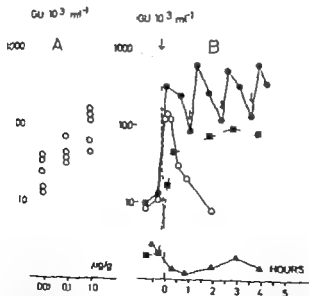


Fig. 1. A. The dose-response relationship of the effect of isoprenaline (in $\mu\text{g/g}$ b.wt.) on renal renin release is shown by the maximum plasma renin concentration (in Goldblatt Units 10^{-6} ml $^{-1}$) in subcutaneously adrenalectomized mice. The mean preinfusion renin concentration was 7.4 ± 1.1 GU 10^{-6} ml $^{-1}$ in 14 mice. After the injection of the 3 doses the mean values were 34.6 ± 4.2 , 46.3 ± 14.8 and 101 ± 20.3 respectively. The difference between the values obtained with the lowest and highest doses being highly significant ($p < 0.005$). The significance between the first and the second group 0.01 vs 0.1 is 0.05 and that between the second and the third group $p < 0.05$. B. Representative examples of the time course of the changes in plasma renin produced by isoprenaline. Single infusion of 1 $\mu\text{g/g}$ b.wt. isoprenaline during one hour (O). Repeated infusions, each of 20 min duration (□). Continuous infusion of 2.6 $\mu\text{g/g}$ h during 4 h (Δ). 1 $\mu\text{g/g}$ isoprenaline given 10 times (Δ).

Differences in renal and submaxillary renin release after stimulation with isoprenaline and noradrenaline

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Abstract

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It is confirmed that while noradrenaline stimulates release of submaxillary as well as renal renin, isoprenaline only stimulates renal renin release. The effects of these two adrenergic agonists differ in several other ways. The kidneys respond to isoprenaline with dose dependant renin release. As a contrast the submaxillary glands respond to noradrenaline by no or by non-dose-dependant release. After a single dose of the agonists the isoprenaline induced renin release is of short duration, contrasting with a prolonged renin release after injection of noradrenaline. The kidneys readily respond to repeated doses of both agonists, while the submaxillary glands most often only respond to the first dose. While the effect of noradrenaline is blocked by pretreatment with phenoxybenzamine, this blocker is without effect when given after noradrenaline. The two agonists do not provoke any increase in plasma renin in mice which have been both adrenalectomized and nephrectomized.

Key words: Adrenergic agents, renin release, plasma renin.

While renal renin release is stimulated by way of beta-adrenergic receptors in the kidney (see Assaykeen, 1972 and Freeman and Davis 1977), in vivo as well as in vitro experiments have shown that release of renin from the submaxillary glands is only stimulated via alpha adrenergic receptors (Menzie *et al* 1974, Michelakis *et al* 1976). The present study aims at investigating the time course of these changes in renal and submaxillary renin release and the dose response relationships for the two agonists, isoprenaline and noradrenaline.

Material and Methods

Animals. Male albino mice of the Danish Stat Serum Institut strain weighing about 50 g, and kept in cages containing 5 or only single mouse. Most mice were conscious, having been placed in restraining cages after insertion of catheters in one or both femoral arteries during a short-term ether anaesthesia. When the agonists were given i.v. (in the jugular vein) the mice were under pentobarbital anaesthesia (60 mg/kg i.p.). In order to obtain basal plasma renin values the experiments were first started about 2 h after ether anaesthesia. Renal renin release was studied a week or more after submaxillary stalk-adrenalectomy and submaxillary release in mice nephrectomized 17 or more fifteen about 2 h advance.

given by the representative curves marked \circ in Fig. 2 B, showing that the effect is prolonged, reaching its maximum after 1 to 2 h and staying at this level for the time of the experiment, lasting up to 6 h. In 8 expts., one of which is given in Fig. 2 B (lowest curve marked \circ), a second dose of 1 $\mu\text{g/g}$ noradrenaline was given 3 h after the first dose, at a time when the maximum level was obtained. The second dose elicited none or only a slight further increase in plasma renin. In a ninth expt. the second dose of 1 $\mu\text{g/g}$ was followed by an increase in plasma renin from about 50 to 400 GU 10^{-3} ml. When a dose of 3 $\mu\text{g/g}$ phenoxylbenzamine was given before noradrenaline there was the usual rise in plasma renin (N 6), but when the dose was increased to 9 $\mu\text{g/g}$ the effect of noradrenaline was blocked (N 6) as shown in the curve marked \bullet . When doses of 3 (N 9) or 9 $\mu\text{g/g}$ phenoxylbenzamine (N 6) were given 1 to 2 h after noradrenaline, there was no fall in the plasma renin concentration, the renin values staying at the high level as shown by the upper curve in Fig. 2 B.

The effect of noradrenaline on renal renin release was studied in 12 previously shalodenectomized mice, of which 7 died of doses between 0.5 and 1 $\mu\text{g/g}$, the pre-treatment with dihydralazine in these mice being omitted because of its hypotensive effect, which causes release of renal (but not of submaxillary) renin. Of the 5 surviving mice one received single injection of 1 $\mu\text{g/g}$ noradrenaline (Δ in Fig. 2 C), while the other 4 received doses from 0.25 to 0.5 $\mu\text{g/g}$, which were repeated after 2 h. As shown in Fig. 2 C the effect of noradrenaline on renal renin release differs from that obtained after infusion of isoprenaline (Fig. 1 B), the effect being prolonged, resulting in an increased level of many hours' duration. And contrary to the most often lacking effect on submaxillary renin release of a second dose of noradrenaline, the second dose resulted in all cases in a marked further increased renal renin release, as shown by representative example marked Δ in Fig. 2 C, in which the second injection is indicated by an arrow marked N. The renin concentration rose from mean preinjection value of 7.8 ± 0.9 to 28.6 ± 5.4 GU 10^{-3} ml ($p < 0.01$) after the first injection (N 5) and from 18.8 ± 2.5 to 95.3 ± 30 ($p = 0.025$) after the second injection (N 4).

II Effect of isoprenaline and noradrenaline on the plasma renin concentration in mice after removal of submaxillary glands as well as kidneys

When the agonists in doses of 1 $\mu\text{g/g}$ were given to mice which after previous shalodenectomy had been nephrectomized about 2 h in advance, it was found, that neither isoprenaline (N 8) nor noradrenaline (N 8) provoked any increase in plasma renin concentration.

Discussion

The results confirm the findings of Menzies *et al.* (1974) and Michelakis *et al.* (1976), showing that the beta-adrenergic agonist isoprenaline causes a release of renal, but not of submaxillary renin, that the alpha-adrenergic agonist noradrenaline causes renin release from both kidneys and submaxillary glands, and that the latter effect of noradrenaline is blocked by phenoxylbenzamine. Both agonists are shown to be unable to provoke an increase in plasma renin concentration in mice, which have been shalodenectomized as well as nephrectomized.

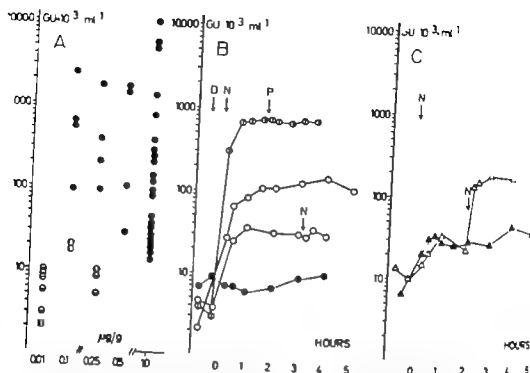


Fig. 2. *A* The effect of different doses of noradrenaline (in $\mu\text{g/g}$) on submaxillary renin release as shown by the maximum plasma renin concentration (in $\text{GU } 10^{-3} \text{ ml}^{-1}$) in 35 nephrectomized mice. \bigcirc mark the cases in which the plasma renin concentration was uninfluenced by the injection and \bullet the cases in which it was higher after injection of noradrenaline than before the injection. There is no dose-response relationship ($p > 0.05$). *B* Representative examples of the time course of the changes in plasma renin concentration after single doses of $1 \mu\text{g/g}$ noradrenaline (arrow marked N) infused during 1 min after pre-treatment with dihydralazine (D) in nephrectomized mice (\bigcirc). In one case (indicated by a second arrow marked N) the infusion was repeated. In the experiment given by (\bullet) $9 \mu\text{g/g}$ of phenylephrine was given before as indicated by the arrow P. *C* The time course of changes in plasma renin concentration due to the renal renin release after a single infusion of $1 \mu\text{g/g}$ noradrenaline in a syngo-adrenalectomized mouse (Δ), and the effect of repeated infusions of doses of $0.25 \mu\text{g/g}$ in a syngo-adrenalectomized mouse (Δ).

arrows) resulted in repeated increases in plasma renin ($N = 7$), and that continued infusion of $2.6 \mu\text{g/g/h}$ during several hours (\equiv) resulted in a rather constant high level ($N = 11$). The figure finally shows that $1 \mu\text{g/g}$ isoprenaline does not cause any release of submaxillary renin when infused in nephrectomized mice ($N = 3$ marked Δ).

II Effect of the predominantly α -adrenergic agonist noradrenaline on release of submaxillary and renal renin

The effect of noradrenaline on the release of submaxillary renin was studied in nephrectomized mice, the doses being between 0.01 and $1 \mu\text{g/g b.wt.}$ given *i.a.* during 1 min after pre-treatment with dihydralazine. The results are seen in Fig. 2 A and B. The maximum value for plasma renin concentration after a single injection of different doses 0.01 , 0.1 , 0.25 , 0.5 and $1 \mu\text{g/g b.wt.}$ is given in Fig. 2 A. It is seen that while a dose of $0.01 \mu\text{g/g}$ was without effect on plasma renin, doses of 0.1 to $1 \mu\text{g/g}$ in most cases resulted in increases with marked differences between the individual maximum values, but without any significant differences between those obtained with the four doses. The time course of the changes in plasma renin

given by the representative curves marked \circ in Fig. 2 B, showing that the effect is prolonged, reaching its maximum after 1 to 2 h and staying at this level for the time of the experiment, lasting up to 6 h. In 8 expts., one of which is given in Fig. 2 B (lowest curve marked \circ), a second dose of 1 $\mu\text{g/g}$ noradrenaline was given 3 h after the first dose, at a time when the maximum level was obtained. The second dose elicited none or only a slight further increase in plasma renin. In a ninth expt. the second dose of 1 $\mu\text{g/g}$ was followed by an increase in plasma renin from about 50 to 400 $\text{GU } 10^{-6} \text{ ml}^{-1}$. When a dose of 3 $\mu\text{g/g}$ phenoxylbenzamine was given before noradrenaline there was the usual rise in plasma renin (4-6), but when the dose was increased to 9 $\mu\text{g/g}$ the effect of noradrenaline was blocked (4-6) as shown in the curve marked \bullet . When doses of 3 (N-9) or 9 $\mu\text{g/g}$ phenoxylbenzamine (N-6) were given 1 to 2 h after noradrenaline, there was no fall in the plasma renin concentration, the renin values staying at the high level as shown by the upper curve in Fig. 2 B.

The effect of noradrenaline on renal renin release was studied in 12 previously sialoadenectomized mice, of which 7 died of doses between 0.5 and 1 $\mu\text{g/g}$, the pre-treatment with dibydrizazine in these mice being omitted because of its hypotensive effect, which causes release of renal (but not of submaxillary) renin. Of the 5 surviving mice one received single injection of 1 $\mu\text{g/g}$ noradrenaline (Δ in Fig. 2 C), while the other 4 received doses from 0.25 to 0.5 $\mu\text{g/g}$, which were repeated after 2 h. As shown in Fig. 2 C the effect of noradrenaline on renal renin release differs from that obtained after infusion of isoprenaline (Fig. 1 B), the effect being prolonged, resulting in an increased level of many hours duration. And contrary to the most often lacking effect on submaxillary renin release of a second dose of noradrenaline, the second dose resulted in all cases in a marked further increased renal renin release, as shown by a representative example marked Δ in Fig. 2 C, in which the second injection is indicated by an arrow marked N. The renin concentration rose from mean pre-injection value of 7.8 ± 0.9 to $28.6 \pm 5.4 \text{ GU } 10^{-6} \text{ ml}^{-1}$ ($p < 0.01$) after the first injection (N-5) and from 18.8 ± 2.5 to 95.3 ± 30 ($p = 0.025$) after the second injection (N-4).

(1) Effect of isoprenaline and noradrenaline on the plasma renin concentration in mice after removal of submaxillary glands as well as kidneys

When the agonists in doses of 1 $\mu\text{g/g}$ were given to mice which after previous sialoadenectomy had been nephrectomized about 2 h in advance, it was found, that neither isoprenaline (N-8) nor noradrenaline (N-8) provoked any increase in plasma renin concentration.

Discussion

The results confirm the findings of Menzie *et al.* (1974) and Michelakakis *et al.* (1976), showing that the beta-adrenergic agonist isoprenaline causes a release of renal, but not of submaxillary renin, that the alpha-adrenergic agonist noradrenaline causes renin release from both kidney and submaxillary glands, and that the latter effect of noradrenaline is blocked by phenoxylbenzamine. Both agonists are shown to be unable to provoke an increase in plasma renin concentration in mice, which have been sialoadenectomized as well as nephrectomized.

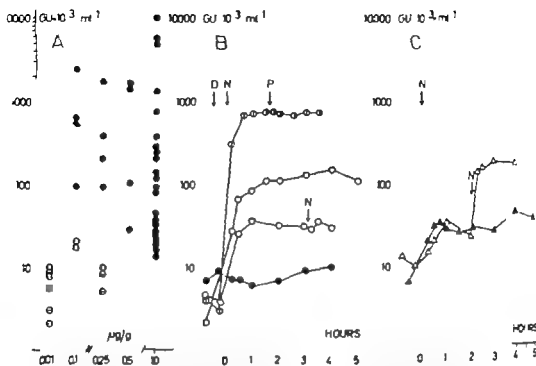


Fig. 2. *A* The effect of different doses of noradrenaline (in $\mu\text{g/g}$) on submaxillary renin release as indicated by the maximum plasma renin concentration (in $\text{GU} \cdot 10^{-3} \text{ ml}^{-1}$) in 35 nephrectomized mice (\bigcirc) and the cases in which the plasma renin concentration was uninfluenced by the injection and (\bullet) the cases in which it was higher after injection of noradrenaline than before the injection. There is no dose-response relationship ($p > 0.05$). *B* Representative examples of the time course of the changes in plasma renin concentration after single doses of $1 \mu\text{g/g}$ noradrenaline (arrow marked N) infused during 1 min after pre-treatment with dihydralazine (D) in nephrectomized mice (\bigcirc). In one case (indicated by a second arrow marked N) infusion was repeated. In the experiment given by (\bullet) $9 \mu\text{g/g}$ of phenoxybenzamine was given before adrenaline. In the experiment marked by (\bigcirc) the same dose was given after noradrenaline at the time indicated by the arrow P. *C* The time course of changes in plasma renin concentration due to the renin release after single infusions of $1 \mu\text{g/g}$ noradrenaline in a sham-operated mouse (Δ), and the effect of repeated infusions of doses of $0.25 \mu\text{g/g}$ in an adrenalectomized mouse (Δ).

arrows) resulted in repeated increases in plasma renin (N-7), and that continued infusion of $2.6 \mu\text{g/g/h}$ during several hours (\bullet) resulted in a rather constant high level (N-7). The figure finally shows that $1 \mu\text{g/g}$ isoprenaline does not cause any release of submaxillary renin when infused in nephrectomized mice (N-3 marked Δ).

II Effect of the predominantly α -adrenergic agonist noradrenaline on release of submaxillary and renal renin

The effect of noradrenaline on the release of submaxillary renin was studied in nephrectomized mice, the doses being between 0.01 and $1 \mu\text{g/g}$ b wt. given i.a. during 1 min after treatment with dihydralazine. The results are seen in Fig. 2 A and B. The maximum values for plasma renin concentration after a single injection of different doses 0.01 , 0.1 , 0.5 and $1 \mu\text{g/g}$ b wt. is given in Fig. 2 A. It is seen that while a dose of $0.01 \mu\text{g/g}$ was without effect on plasma renin, doses of 0.1 to $1 \mu\text{g/g}$ in most cases resulted in increases with marked differences between the individual maximum values, but without any significant differences between those obtained with the four doses. The time course of the changes in plasma renin

given by the representative curves marked \square in Fig. 2 B, showing that the effect is prolonged, reaching its maximum after 1 to 2 h and staying at this level for the time of the experiment, lasting up to 6 h. In 8 expts., one of which is given in Fig. 2 B (lowest curve marked \circ), a second dose of 1 $\mu\text{g/g}$ noradrenaline was given 3 h after the first dose, at a time when the maximum level was obtained. The second dose elicited none or only a slight further increase in plasma renin. In a ninth expt. the second dose of 1 $\mu\text{g/g}$ was followed by an increase in plasma renin from about 50 to 400 $\text{GU} \cdot 10^{-6} \cdot \text{ml}^{-1}$. When a dose of 3 $\mu\text{g/g}$ phenylephrine was given before noradrenaline there was the usual rise in plasma renin (Fig. 6), but when the dose was increased to 9 $\mu\text{g/g}$ the effect of noradrenaline was blocked (Fig. 6) as shown in the curve marked \bullet . When doses of 3 (N = 9) or 9 $\mu\text{g/g}$ phenylephrine (N = 6) were given 1 to 2 h after noradrenaline, there was no further increase in plasma renin concentration, the renin values staying at the high level as shown in the curve in Fig. 2 B.

The effect of noradrenaline on renal renin release was examined in mice, of which 7 died of doses between 0.5 and 1.0 $\mu\text{g/g}$, with dihydralazine in these mice being omitted because of massive release of renal (but not of submaxillary) renin. Of the 5 surviving mice a single injection of 1 $\mu\text{g/g}$ noradrenaline (Δ in Fig. 2 C), or 0.25 to 0.5 $\mu\text{g/g}$, which were repeated after 2 h. As compared with the effect of noradrenaline on renal renin release differs from that obtained with phenylephrine (Fig. 1 B), the effect being prolonged, resulting in an increase in plasma renin. And contrary to the most often lacking effect on submaxillary renin release of noradrenaline, the second dose resulted in all cases in a further increase in renal renin release, as shown by a representative example in Fig. 2 C. The mean preinjection value of 7.8 ± 0.9 to $28.6 \pm 5.4 \text{ GU} \cdot 10^{-6} \cdot \text{ml}^{-1}$ after the first injection (N = 5) and from 18.8 ± 2.5 to 95.3 ± 30 ($p = 0.025$) after the second injection (N = 4).

11 Effect of isoprenaline and noradrenaline on the plasma renin concentration of submaxillary glands as well as kidneys

When the agonists in doses of 1 $\mu\text{g/g}$ were given to mice which after preadrenalectomy had been nephrectomized about 2 h in advance, it was found, that neither isoprenaline (N = 5) nor noradrenaline (N = 5) provoked any increase in plasma renin concentration.

Discussion

The results confirm the findings of Menzie *et al.* (1974) and Michelakis *et al.* (1974) that the beta-adrenergic agonist isoprenaline causes a release of renal, but not of submaxillary renin, that the alpha-adrenergic agonist noradrenaline causes renin release from both kidneys and submaxillary glands, and that the latter effect of noradrenaline is blocked by phentolamine. Both agonists are shown to be unable to provoke an increase in plasma renin concentration in mice, which have been adrenalectomized as well as in

This finding shows the correctness of the assumption of Menzie *et al.* (1974), that the creases in renin concentration in sialoadenectomized and in nephrectomized mice effected by the two agonists were due to release of renal and submaxillary renin respectively.

It is further shown that the effects of these two adrenergic agonists differ in several ways. While there is a rather good dose-response-relationship of the effect on plasma renin concentration after injection of isoprenaline on sialoadenectomized mice with intact livers (Fig. 1 A), no such relationship is seen in noradrenaline treated nephrectomized mice with intact submaxillary glands, as shown in Fig. 2 A, which further shows the marked differences in the individual responses to noradrenaline.

Further differences are seen in the time course of the changes in plasma renin concentration that produced by isoprenaline (due to renal renin release) being short (Fig. 1 B), while noradrenaline causes a change in submaxillary renin release, resulting in a prolonged increase in plasma renin, lasting more than 6 h. This continued high level can be due to continued release of submaxillary renin. The finding that doses of phenoxylbenzamine, which block the effect of noradrenaline, when given before this agonist, are without effect, when given after the noradrenaline (Fig. 2 B), could be due to a similar mechanism, as that case of the similarly continued high level found after gentle manipulation and subsequent removal of the submaxillary glands in nephrectomized mice. In these it was found that the continued high level of plasma renin concentration was due to transfer of renin with submaxillary lymph or interstitial fluid (perhaps with renin containing tubular granules) to surrounding tissues with a subsequent slower release from these tissues to the blood (Bing *et al.* 1977). The lack of effect of phenoxylbenzamine on the high renin level when given after noradrenaline, can, however, also be due either to inability of the phenoxylbenzamine to displace the agonist from the receptors, or more likely to an inability of the blocker to influence the secretion after the latter has been stimulated. The prolonged increase in plasma renin which was found after a single injection of noradrenaline both in the nephrectomized (Fig. 2 B) and in the sialoadenectomized mice (Fig. 2 C) can, however, also be due either to release of inactive renin, which is activated with time, or to a protracted release due to severe circulatory stress caused by treatment with noradrenaline.

Renal and submaxillary renin differ in their ability to respond to repeated doses of adrenergic agonists. The renal renin was released each time isoprenaline (Fig. 1 B) or noradrenaline (Fig. 2 C) was given while submaxillary renin in all but one case was only released following the first dose (Fig. 2 B). This difference may be related to the finding of the marked individual, non-dose dependent responses of submaxillary renin release (Fig. 2 A), which contrasts to the dose-dependency of the response of renal renin release to isoprenaline (Fig. 1 A).

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References

1. HAMAYAN, T. A. *Control of Renin Secretion*. Plenum Press, New York 1972, especially p. 17 and 49.
2. ICHIO, J. and K. FOLLMEY, Participation of renal, but not of submandibular renin in the homeostasis of the blood pressure after experimentally induced hypotension in mice. *Acta path. microbiol. scand.* 1976, 84 A 391-396.
3. ICHIO, J. C. MALLINO and K. FOLLMEY, Cause of the continuous rise in plasma renin concentration after removal of encapsulated submandibular glands in nephrectomized mice. *Acta path. microbiol. scand.* 1977, 85 A 683-690.
4. BLUMAN, R. H. and J. H. DAVIS, Control of renin secretion and metabolism. In: GINSBURG J. E. KROW and E. KOCHER, *Hypertension*. McGraw Hill, New York 1977 p. 210.
5. MENZER, J. W. A. M. MICHELAKIS, and H. YOSHIDA, Sympathetic nervous system and renin release from submandibular glands and kidneys. *Amer J Physiol.* 1974, 227 1281-1284.
6. MICHELAKIS, A. M. J. W. MENZER and H. YOSHIDA, Sympathetic nervous system and renin release from submandibular glands in dogs. *Amer J Physiol.* 1976, 231 551-554.
7. FOLLMEY, K. and J. JOSEFSSON, An easy radioimmunochemical microassay of renin activity concentration and secretion in human and animal plasma and tissues based on angiotensin I trapping by antibody. *J Clin Endocrinol. Metab.* 1974, 39 816-823.

**In mice aggressive behaviour provokes vast increase
in plasma renin concentration, causing only slight, if any,
increase in blood pressure**

By

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Abstract

BING J and K. POULSEN *In mice aggressive behaviour provokes vast increase in plasma renin concentration causing only slight if any increase in blood pressure* Acta physiol. scand. 1979 105 64-72.

In mice aggressive behaviour causes a vast release of renin, which can result in about 600-fold increase in plasma renin concentration, reaching 6 Goldblatt Units, corresponding to 15 μg renin per ml. This increase is mainly due to release of submaxillary renin, but there is also a significantly increased renal renin release. The degree of renin release is influenced by the duration of the aggression and by previous contact with other mice. Contrasting with the vast increase in plasma renin the blood pressure is normal or only moderately increased. This disproportion is not due to the depletion of renin substrate caused by the increased renin, as shown by the increased calculated renin activity as well as by decrease in blood pressure elicited by blockade of the renin system. Nor is the disproportion due to change in the sensitivity of the vessel to angiotensin II, the cause of this lack of tachyphylaxis being unknown. By way of exclusion the lack of pronounced decrease in blood pressure can be explained by homeostatic function of the cardiovascular reflexes, which may also account for the fact that the pressor response after injection of pure submaxillary renin is only short, contrasting with a prolonged marked increase in plasma renin concentration.

Key words. Aggressive behaviour renin release blood pressure

Renal renin release is known to be influenced by several factors (see Freeman and Daw 1977), and in mice renin is released from the submaxillary glands by stimulation with alpha-adrenergic agonists (Menzie *et al* 1974). It is, therefore, necessary to control the circumstances under which blood is collected for determination of basal plasma renin concentration and activity as rigorously as possible (see Pettinger *et al* 1975). In mice we have previously aimed at having "standard conditions" by using conscious mice kept in restrained cages after insertion of a catheter in a femoral artery during a short-term ether anaesthesia. This pretreatment produced a primary increase in plasma renin concentration, which generally returned to "basal values" after about 2 to 3 h (Bing and Poulsen 1975). Some of these mice, however, had markedly higher renin concentration, the cause of which was unknown. In continued experiments the occurrence of such exceptions was confirmed.

As being the case in normal, nephrectomized as well as in sialoadenectomized mice, An effect of the cause of these exceptional cases with high values was obtained when plasma renin concentrations about 20 to 200-fold the usual basal values were found in 3 mice, which had been kept in social contact and which had been vigorously fighting before the start of the experiment. Based on this observation the aim of the present investigation was to study the effect of aggressive behaviour on the release of renal and submaxillary renin. The aggression was caused by social contact between normal, sialoadenectomized or nephrectomized previously individually housed mice. For comparison the plasma renin concentration was determined in isolated mice, which had not been confronted with other mice. Besides plasma renin and renin substrate concentration, renin activity and blood pressure were determined in several of the mice. In some of these the response of the cardiovascular system to angiotensin II and noradrenaline as well as the effect of blockade of the renin system on the blood pressure were also studied.

Material and Methods

Mice. Male albino mice of the Danish State Serum Institute strain, weighing 50–70 g, individually caged in cages (internal size: 27 × 13 × 9.5 cm) from 2 to 30 days when used for determination of plasma renin concentration, the differences in time of isolation being without influence on the values. Most mice are conscious during sampling of arterial blood as described in the introduction, but in some mice blood could not be sampled during 1–2 min lasting light ether anaesthesia, the type of sampling had no palpable influence on the results.

Aggressive behaviour was obtained by grouping 3 mice, each had previously been housed individually for at least 1 month. A pre-fighting between the animals had previously been provoked (after 14 days isolation), as this has been found to intensify the aggressive behaviour (Kruuk and Jenks 1969). The grouping took place in small cages (13 × 13 × 9.5 cm), in which the mice were kept for varying times, as described in the following, the fighting behaviour being recorded in all experiments (see Vahelli 1969 and Lagerstedt 1969). In some experiments groups of 3 mice were placed in smaller small cages for 10 min three times each for at least 1 month before their aggressive behaviour and its influence on renin release and blood pressure were tested. Some mice were normal, others had been submaxillary sialoadenectomized at least one and most often several weeks before, and a few mice were nephrectomized during short-term ether anaesthesia 2 h before the grouping.

Determination of plasma renin concentration and renin substrate and calculation of the plasma renin activity were performed as described in recent paper (Bang and Poulsen 1977), using the capillary radioimmunoassay of Fomon and Jørgensen (1974). Conversion of Goldblatt Units 10^{-3} (expressed by comparison with the Holly Hill hog serum standard, London) to ng renin was performed by multiplication with 44, based on the finding that the specific enzymatic activity of submaxillary and renal renin is 0.41 10^{-3} Goldblatt Unit 1 ng^{-1} (Malling and Poulsen 1977 b). Blood pressure was recorded with Tybjaerg Hansen ramnator and Servogor 511 recorder. The pure submaxillary renin used for injection, was that purified by Malling and Poulsen (1977). Blockade of the angiotensin system was performed by a infusion of 3 mg/kg/h of the competitive angiotensin II inhibitor Saralasin (Norwich Pharma, Corp.). Response of the cardiovascular system to angiotensin II was estimated by determination of the blood pressure responses to synthetic Asp¹-D²-ang. II (Schwartz Bio Res.) which were compared with those elicited by noradrenaline.

Results

Plasma renin concentration in mice which are individually housed in order to avoid aggressive behaviour. When given in Goldblatt Units 10^{-3} ml the mean plasma renin was 9.8 ± 5.1 in 17 normal, 7.9 ± 3.2 in 25 at least 1 week earlier sialoadenectomized, 3.5 ± 1.7 in 16 mice 2 h after nephrectomy and about the same 3.1 ± 1.8 in 10 mice nephrectomized

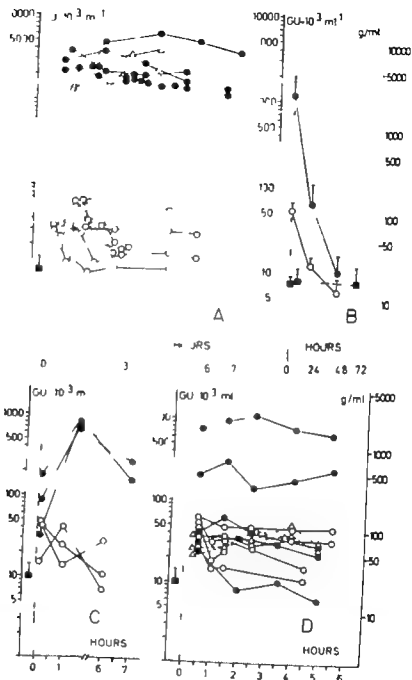


Fig. 1 A The time course of the plasma renin concentrations after prolonged (30 min) aggressive behaviour in normal (●), nephrectomized (Δ) and subcutaneous adrenalectomized (○) mice during the first 3 to 7 h. These increases are all significant ($P < 0.001$). B. Mean values from similarly treated mice (6 normal and 6 subcutaneous adrenalectomized) during 48 to 72 h. The fig. includes the mean values from 3 normal mice not provoked to aggression (■). The lines connecting the values are drawn without consideration of the primary plateau seen in Fig. 1 A. C. Plasma renin concentrations observed after only 3 min aggressive behaviour. D. Responses of undrugged mice which before prolonged aggressive behaviour had been treated for about a month with repeated confrontations.

The symbols are the same in all figures. The left ordinate indicates the plasma renin concentrations GU 10^{-3} ml $^{-1}$ and the right ordinate the same values after conversion to g/ml given on a logarithmic scale. The abscissae show the time in hours after the start of the confrontation, which is marked by a vertical dotted line.

h earlier and xialo-adenectomized at least 1 week earlier. Two values which differed markedly from the mean concentrations were excluded, 59 GU 10^{-3} ml $^{-1}$ found in sera of a normal mouse and 39 found in that of a xialo-adenectomized mouse.

2. *Effect of prolonged pronounced aggressive behaviour on plasma renin concentration and subsequent changes in plasma renin substrate concentration and renin activity* During grouping or 30 min of previously isolated mice the type of aggressive behaviour was the same in normal and xialo-adenectomized mice. The fighting behaviour exploded immediately with powerful attacks, which were repeated up to 5 times a minute or consisted of up to 20 s lasting fight, most often including all 3 mice. In the last 10 min the attacks were less fierce and other types of the aggressive pattern, such as nosing, tail rattling and squeaking were observed. In the nephrectomized mice the aggressive behaviour started with these types, but after 2 min the first real attack was observed. The aggression was, however less pronounced in these mice.

In 10 normal mice the aggressive behaviour caused a vast increase in plasma renin concentration, which was about 100 to 600-fold the normal and stayed at these high levels or 6 h (● Fig. 1 A). As shown by the mean values (● in Fig. 1 B) from 6 other normal mice studied for 48 h after the aggression, normal values were first reached after about 42 h. In 3 xialo-hours previously nephrectomized mice (Δ Fig. 1 A) the renin concentration was only questionably lower than after aggression in the normal mice. 8 xialo-adenectomized mice had also increased renin concentration, which, however was only about 2–10 times the normal values. In several of these mice the renin concentration declined during the 3 to 6 h lasting expts. (○ Fig. 1 A) As shown by the mean values (○ in Fig. 1 B) from 6 other xialo-adenectomized mice normal values were reached after about 24 h. Fig. 1 B further shows the mean of values from 3 normal mice, which had not been made aggressive (marked #). These values agree with the above mentioned mean from 17 other normal mice.

The effect of the vast aggression-provoked increase in plasma renin concentration on plasma renin substrate and renin activity was studied in 8 of the normal mice at the end of the experiments shown in Fig. 1 A. While the mean renin substrate concentration in 8 normal controls was 363 ng ml $^{-1}$ AI (range 202–448), there was a marked depletion of the substrate in the aggressive mice, in which the mean value was 58 (range 24–90). The corresponding calculated mean renin activity was 3 ng AI/ml/h (range 1.2–4.7) in the 8 controls and 17 (range 4.5–29.5) in the 8 experimental mice, in which it was determined about 5 h after prolonged aggression. An other sign of presence of angiotensin in the blood as obtained by blockade of the renin system with the competitive angiotensin II inhibitor Saralasin (Fig. 2 D). In 5 normal mice, which after prolonged aggressive behaviour had not increase in plasma renin concentration (mean: 1350 GU 10^{-3} ml $^{-1}$), Saralasin infusion resulted in a mean decrease of the blood pressure of 23 mmHg (range 11 to 32 mm).

3. *Effect of short pronounced aggressive behaviour on plasma renin concentration* was studied in 3 normal and in 3 xialo-adenectomized mice. In these studies pretreatment and grouping as performed as described above, but the animals were separated after 3 min fighting. In the 3 normal mice the plasma renin concentration was markedly increased immediately after the aggression (Fig. 1 C) and still much higher 90 min later at which time they however did not reach the values found after prolonged aggression (Fig. 1 A). Contrary to the

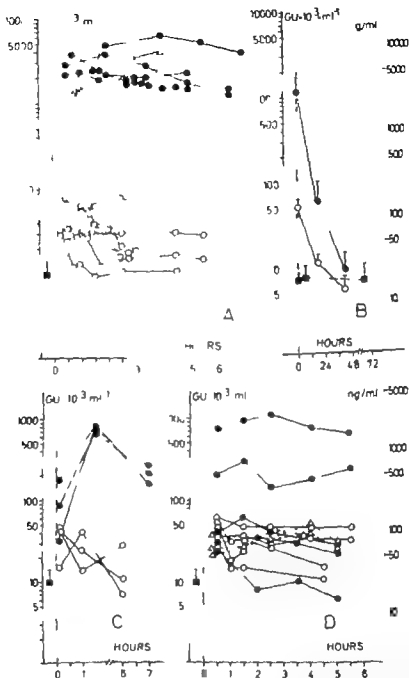


Fig. 1. A. The time course of the plasma renin concentrations after prolonged (30 min) aggressive behaviour in normal (●), nephrectomized (Δ) and submaxillary unloaded/nephrectomized (○) mice during 3 and 7 h. These increases are all significant ($P < 0.001$). B. Mean values from similarly treated mice (6 normal and 6 subloadenectomized) during 48 to 72 h. The fig. includes the mean values from 3 normal mice not provoked to aggression (■). The lines connecting the values are drawn without consideration of the primary plateau seen in Fig. 1 A. C. Plasma renin concentrations observed after only 3 min aggressive behaviour. D. Responses found in mice which before prolonged aggressive behaviour had been pre-treated for about a month with repeated confrontations.

The symbols are the same in all figures. The left ordinate indicates the plasma renin concentrations in $\text{GU} \cdot 10^{-3} \text{ ml}^{-1}$ and the right ordinate the same values after conversion to g/ml given on logarithmic scale. The abscissa show the time in hours after the start of the confrontation, which is marked by vertical dotted line.

h earlier and also adrenalectomized at least 1 week earlier. Two values which differed markedly from the mean concentrations were excluded, 59 GU 10^{-3} ml found in sera of normal mouse and 39 found in that of a adrenalectomized mouse.

Effect of prolonged pronounced aggressive behaviour on plasma renin concentration and consequent changes in plasma renin substrate concentration and renin activity During grouping

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3 Effect of short pronounced aggressive behaviour on plasma renin concentration was studied in 3 normal and in 3 adrenalectomized mice. In these studies pretreatment and grouping was performed as described above, but the animals were separated after 3 min fighting. In the 3 normal mice the plasma renin concentration was markedly increased immediately after the aggression (Fig. 1 C) and still much higher 90 min later at which time they however did not reach the values found after prolonged aggression (Fig. 1 A). Contrary to the

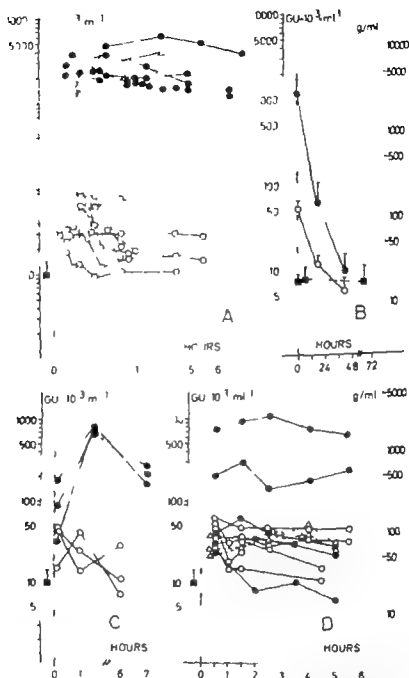


Fig. 1. *A* The time course of the plasma renin concentrations after prolonged (30 min) aggressive behaviour in normal (●), nephrectomized (Δ) and submaxillary aladonectomized (○) mice during the first 3 to 7 h. These increases are all significant ($P < 0.001$). *B* Mean values from similarly treated mice (6 normal and 6 aladonectomized) during 48 to 72 h. The line includes the mean values from 3 normal mice not provoked to aggression (■). The lines connecting the values are drawn without consideration of the primary plateau seen in Fig. 1*A*. *C* Plasma renin concentrations observed after only 3 min aggressive behaviour. *D* Responses found in mice which before prolonged aggressive behaviour had been pre-treated for about a month with repeated confrontations.

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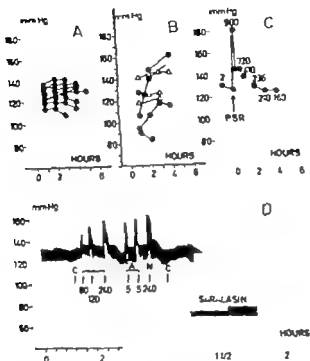


Fig. 2. *A.* Blood pressures in normal, non-pre-treated mice and *B.* blood pressures after prolonged aggressive behaviour in normal (\bullet) and adrenalectomized (Δ) mice with vast increases in plasma renin concentration (as shown in Fig. 1 *A*). *C.* The effect on the blood pressure of submaxillary renin (P.S.R.); the corresponding plasma renin concentrations are given in O.U. 10^{-4} ml $^{-1}$ by figures at the curve. *D.* Blood pressure tracing in mice with vast increase in plasma renin after prolonged aggressive behaviour. Pressure responses to therapy for angiotensin II (A, doses given in μg) and norepinephrine (N, doses in μg). C indicates control phases of physiological saline. The latter part of the tracing shows the depressor effect of Saralasin in mice (initially 3, later 6 μg kg^{-1} hr $^{-1}$).

since the mean blood pressure was 125 mmHg (range 108–145) with only small (max. 10 mm) variations with time in the individual mouse.

For comparison with the relation between plasma renin concentration and blood pressure in aggressive mice the same relation was investigated in 5 adrenalectomized and nephrectomized mice, in which the two parameters were measured before and after intraarterial injection of pure submaxillary renin (P.S.R.). A representative example of the nearly identical results of these experiments is given in Fig. 2 *C*. Although the renin concentration, as shown by the figures given at the curve, rose from 2 to 900 GU 10^{-4} ml $^{-1}$ and was still about 80-fold increased after 4 h, a marked increase in blood pressure was only present for about 15 min. It was below the upper limit of the normal (140 mmHg) after 1 h, reaching the pre-injection level about 2 h after the injection, at a time when the renin concentration was still well over 100-fold increased.

Fig. 6. *Cardiovascular reactivity to the pressor action of angiotensin II in mice with vast increase in plasma renin concentration and activity caused by aggressive behaviour.* 5 normal mice, in

constant high values found after prolonged aggression, the values had fallen marked the end of the 7 h lasting experiment. In the 3 sialoadenectomized mice the short aggression resulted in an increase in plasma renin, which was similar to the lowest values found at prolonged aggression.

4 *The influence of repeated confrontations on the type of aggressive behaviour and on plasma renin concentration* was studied in 9 normal mice, one of which died when it was anaesthetized after the last confrontation and in 6 sialoadenectomized mice.

After 3 weeks isolation groups of 3 of these mice were brought in contact for 10 min three times a week for 4 to 6 weeks. At the start of this period the type of aggressive behaviour was the same as previously described, but in all 9+6 mice the aggressiveness declined with time. This was especially the case in the normal mice, which for the last half of the month started with nosing and tail rattling, powerful attacks first taking place after 30-5 min contact. Even then the attacks were less frequent and were of shorter duration than in the first confrontations. After this pretreatment the 3 mice of each group were confronted for about 20 min during which the level of aggression was decreased as in their last fighting. 3 of the normal mice had been nephrectomized 2 h before this confrontation. In these there was no signs of aggression, and even addition of 2 normal mice elicited only a few attacks.

The effect of this decreased aggression on plasma renin concentration (Fig. 1 D) was about the same in the sialoadenectomized (\circ) nephrectomized (Δ) and in 3 of the 5 normal (\bullet) mice, the values being between 25 and 60 GU 10^{-4} ml. In two of the normal mice higher values were found but they did not reach the level observed following aggression in normal mice, not subjected to the above mentioned pretreatment (\bullet Fig. 1 A).

5 *Effect of the vast increase in plasma renin concentration on the blood pressure* The blood pressure was followed for up to 4 h in 5 of the normal and in the 3 nephrectomized mice in all of which the renin concentration after the aggression rose to and stayed above 100 GU 10^{-4} ml as shown in Fig. 1 A (\bullet and Δ). While the blood pressure of 10 control mice with normal plasma renin was between 110 and 140 mmHg with only small change (maximum 10 mm) with time (Fig. 2 A), the blood pressure of the 8 mice with vast increase in plasma renin was more variable, as seen in Fig. 2 B (in which normal mice are marked \circ nephrectomized Δ). This was both due to more varying blood pressure values with time as to more marked individual differences. The blood pressure did, however, only exceed the maximum level of the normal (140 mmHg) in three mice, in which the values reached 145, 154 and 160 mm respectively.

The time course of the blood pressure of sialoadenectomized mice was studied in the 6 mice in which aggressive behaviour had resulted in renin concentrations from about 25 to 100 GU 10^{-4} ml (\circ Fig. 1 A). The mean blood pressure of these mice was identical with that of 7 sialoadenectomized mice, which had not been subjected to aggression, the mean values in both being 123 mmHg (range 110-140) with only small (max. 12 mm) or any variation with time in the individual mouse.

The time course of the blood pressure was finally followed for 4 h in 3 of the normal and the 3 sialoadenectomized mice after the aggression which followed the repeated fighting 3 times a week during 4 to 6 weeks, the renin values of which are shown in Fig. 1 D. In the

3. *Effect of the fast increase in plasma renin concentration on blood pressure* The blood pressure of normal isolated mice was between about 110 and 140 mmHg (Fig. 2 A), not differing from that previously found in mice kept in social contact (Bling and Poulsen 1975). In pronounced contrast to the vast increases in plasma renin concentration found after aggression the blood pressures were only somewhat above the maximum level of the normal few mice. However the blood pressure was more variable with time and between individuals (Fig. 2 B). The marked disproportion between plasma renin and blood pressure similar to that previously found after manipulation of the submaxillary glands (Bling and Poulsen 1977), and its cause can be the same in these two groups of differently pre-cared mice. Also in the present study the only small, if any increase in blood pressure could not be attributed to a demonstrated significant depletion of renin substrate, as this was actually associated with a marked increase in calculated renin activity. A direct demonstration of formation and function of angiotensin II in the post-aggressive period was obtained by the finding of a significant decrease in blood pressure after blockade of the renin-system with the competitive angiotensin II inhibitor Saralasin (Fig. 2 D). The disproportion between plasma renin and blood pressure was further found not to be due to altered responsiveness (tachyphylaxis) of the resistance vessels to angiotensin II, as the animals showed a normal response to angiotensin II (Fig. 2 D), a surprising finding in animals with such fast increases in plasma renin concentration. Such lack of tachyphylaxis was also found in about half of the mice with similarly high renin values after manipulation of the submaxillary glands. After aggression as well as after manipulation, release of submaxillary kallikrein or another depressor substance which could counteract the effect of the increased release of renin, cannot be excluded. That disproportion between renin and blood pressure occurs even in experiments, in which release of such a hypothetical submaxillary depressor substance can be excluded, is shown by the experiments on alloxan-treated as well as nephrectomized mice, in which injection of pure submaxillary renin resulted in prolonged marked increase in plasma renin with an only short-lasting increase in blood pressure (Fig. 2 C). As the disproportion between plasma renin and blood pressure could not be explained in any of the previously discussed ways it must for the present be believed that the lack of increase in blood pressure is due to homeostatic function of slow compensatory cardiovascular reflexes (see Day et al. 1965). However such compensation does not make itself apparent after application of a Goldblatt clamp where an increase in plasma renin is accompanied by a marked increase in blood pressure.

This study was supported by grants from the Danish Heart Foundation, King Christian X. Foundation and the Foundation of the Insurance Companies of 1952. The authors are also grateful to Dr Alan W. Castellan, The Norwich Pharmacal Co. New York for the generous gift of Saralasin. Dr Ole Frederiksen kindly performed the statistical calculations.

References

1. Bling, J. and K. Poulsen, Different effects on renal and submaxillary renin release after blockade of the renin system in mice. *Acta path. Microbiol. scand.*, sect. A. 1975. 83: 733-736.
2. Bling, J. and K. Poulsen, Cause of the disproportion between the fast increase in plasma renin and the only small (if any) increase in blood pressure after manipulation of the submaxillary glands. *Acta path. Microbiol. scand.* sect. A. 1977. 85: 691-700.

which aggression had resulted in a mean plasma renin concentration of $1\ 350\ \text{GU}\cdot 10^{-4}\cdot \text{ml}^{-1}$ and 2 *sialo*adenectomized mice with less increased concentrations (48 and 86 $\text{GU}\cdot 10^{-4}\cdot \text{ml}^{-1}$) reacted with a pressor response of about 25 mmHg, when 5 ng angiotensin II was injected the equipressor dose of noradrenaline being 130 to 240 ng (*cf.* Fig. 2D). This response is about the same as that found in 6 normal isolated mice, which had not been made aggressive. In these a pressor response of about 25 mm was obtained with 2.5 to 5 ng angiotensin II the equipressor response being obtained with 240 to 480 ng noradrenaline.

Discussion

1 *The plasma renin concentrations of individually housed mice* were found to be the same as those previously found in mice kept in social contact (Bing and Poulsen 1975) the concentrations in the *sialo*adenectomized being insignificantly lower than in the normal while markedly lower values were found in nephrectomized mice and after removal of kidneys as well as submaxillary glands. Abnormally high values were more seldom found in isolated mice than in mice kept in social contact the few high values found showing that other causes than aggression can result in increased renin release.

After conversion of the concentrations of enzymatically active renin from Goldblatt units to ng renin per ml the mean values in normal and *sialo*adenectomized mice were 24 to 11 respectively which agrees well with the values of 23 and 21 ng found by Menzies *et al.* (1974) by radioimmunoassay for the direct measurement of renin. 17 h after nephrectomy of 11 previously *sialo*adenectomized mice the calculated mean concentration was 7.5 ng/ml which is lower than the mean value of 11 ng/ml found by Menzies *et al.* in 10 mice 24 h after removal of submaxillary glands as well as kidneys.

2 *Effect of aggressive behaviour on renal and submaxillary renin release* The study shows that prolonged aggressive behaviour in mice results in vast increase in plasma renin concentration reaching values between $1\ 500$ and $6\ 000\ \text{GU}\cdot 10^{-4}\cdot \text{ml}^{-1}$ (corresponding to 3.7 to 15 $\mu\text{g}/\text{ml}$) and thus well over 150 to 600-fold the normal concentration (\bullet in Fig. 1A). These maximum values are mainly due to release of submaxillary renin, as shown by the only little lower concentrations found in nephrectomized mice (Δ in Fig. 1A), by the markedly lower concentrations found in *sialo*adenectomized mice (\circ in Fig. 1A) and by our previous finding of a similar vast increase after gentle manipulation of the submaxillary glands (Bing and Poulsen 1977). That the aggression also causes release of renal renin is made probable in the experiments on *sialo*adenectomized mice (\circ Fig. 1A) the plasma renin here being about 2 to 10-fold increased. In these mice participation of an extra-renal release can however not be excluded.

The high values, are especially in the normal and nephrectomized mice, rather constant for many hours. The subsequent fall is slow normal values not being reached till after about 42 h (Fig. 1B). Increased plasma renin concentration was also found immediately after only few minutes aggression the values in normal mice increasing further to about $1\ 000\ \text{GU}\cdot 10^{-4}$ and thereafter again decreasing, as shown in Fig. 1C. After repeated confrontations resulting in aggressive behaviour 3 times a week during at least 1 month both the aggression and its influence on plasma renin concentration were less marked (Fig. 1D), this in the mice, in which such pretreatment was omitted (Fig. 1A).

3. *Effect of the sex increase in plasma renin concentration on blood pressure* The blood pressure of normal isolated mice was between about 110 and 140 mmHg (Fig. 2 A), not differing from that previously found in mice kept in social contact (Bing and Poulsen 1975). In pronounced contrast to the vast increases in plasma renin concentration found after aggression the blood pressures were only somewhat above the maximum level of the normal in few mice. However the blood pressure was more variable with time and between individuals (Fig. 2 B). The marked disproportion between plasma renin and blood pressure is similar to that previously found after manipulation of the submaxillary glands (Bing and Poulsen 1977), and its cause can be the same in these two groups of differently pre-treated mice. Also in the present study the only small, if any increase in blood pressure could not be attributed to a demonstrated significant depletion of renin substrate, as this was actually associated with a marked increase in calculated renin activity. A direct demonstration of formation and function of angiotensin II in the post-aggressive period was obtained by the finding of a significant decrease in blood pressure after blockade of the renin-system with the competitive angiotensin II inhibitor Saralasin (Fig. 2 D). The disproportion between plasma renin and blood pressure was further found not to be due to reduced responsiveness (tachyphylaxis) of the resistance vessels to angiotensin II as the animals showed a normal response to angiotensin II (Fig. 2 D), a surprising finding in animals with such vast increases in plasma renin concentration. Such lack of tachyphylaxis is also found in about half of the mice with similarly high renin values after manipulation of the submaxillary glands. After aggression as well as after manipulation, release of submaxillary kallikrein or another depressor substance which could counteract the effect of the increased release of renin, cannot be excluded. That disproportion between renin and blood pressure occurs even in experiments, in which release of such a hypothetical submaxillary depressor substance can be excluded, is shown by the experiments on alodendec treated as well as nephrectomized mice, in which injection of pure submaxillary renin resulted in prolonged marked increase in plasma renin with an only short-lasting increase in blood pressure (Fig. 2 C). As the disproportion between plasma renin and blood pressure could not be explained in any of the previously discussed ways it must for the present be believed that the lacking increase in blood pressure is due to homeostatic function of slow compensatory cardiovascular reflexes (see Day *et al.* 1963). However such compensation does not make itself apparent after application of a Goldblatt clamp, whence an increase in plasma renin is accompanied by marked increase in blood pressure.

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References

- BING, J and K. POULSEN, Different effects on renal and submaxillary renin release after blockade of the renin system in mice. *Acta path. Microbiol. scand. sect. A.* 1975. 83: 733-736.
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which aggression had resulted in a mean plasma renin concentration of $1\ 350\ \text{GU} \times 10^{-4}\ \text{ml}^{-1}$ and 2 sialoadenectomized mice with less increased concentrations (48 and 86 $\text{GU} \times 10^{-4}\ \text{ml}^{-1}$) reacted with a pressor response of about 25 mmHg, when 5 ng angiotensin I was injected, the equipressor dose of noradrenaline being 130 to 240 ng (cf. Fig. 2D). This response is about the same as that found in 6 normal isolated mice, which had not been made aggressive. In these a pressor response of about 25 mm was obtained with 2.5 to 5 ng angiotensin II the equipressor response being obtained with 240 to 480 ng noradrenaline.

Discussion

1. *The plasma renin concentrations of individually housed mice* were found to be the same as those previously found in mice kept in social contact (Bing and Poulsen 1975), the concentrations in the sialoadenectomized being insignificantly lower than in the normal while markedly lower values were found in nephrectomized mice and after removal of kidney as well as submaxillary glands. Abnormally high values were more seldom found in isolated mice than in mice kept in social contact the few high values found showing that other causes than aggression can result in increased renin release.

After conversion of the concentrations of enzymatically active renin from Goldblatt unit to ng renin per ml the mean values in normal and sialoadenectomized mice were 24 and 21 respectively which agrees well with the values of 23 and 21 ng found by Menzie *et al.* (1974) by radioimmunoassay for the direct measurement of renin 17 h after nephrectomy of 11 previously sialoadenectomized mice the calculated mean concentration was 7.5 ng/ml which is lower than the mean value of 11 ng/ml found by Menzie *et al.* in 10 mice 24 h after removal of submaxillary glands as well as kidneys.

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Capillary permeability measured by bolus injection, residue and venous detection

By

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Abstract

SEJSEN, P. *Capillary permeability measured by bolus injection, residue and venous detection.*
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Micro analysis of residue and outflow curves of γ -emitting indicators such as chromium-51 EDTA and deuterium-131-thalamate from skeletal muscle gives the possibility to determine the extraction fraction and in plasma flow and from these two values the capillary diffusion capacity can be calculated (Sejnsen 1970, preliminary report). This is possible both for the transport from blood to tissue and from tissue to blood. This alternative method has been compared in the autoperfused cat gastrocnemius preparation with the indicator diffusion method based on venous registration of diffusible test indicator and an intravascular reference indicator (Chinard *et al.* 1955, Crone 1963). The results of the five independent measurements show good agreement. Calculation of the permeability P_d based on capillary surface area 17 000 cm²/100 g of tissue gives values of $1.65 \cdot 10^{-6}$, $1.10 \cdot 10^{-6}$ and $1.16 \cdot 10^{-6}$ cm/s, which is in agreement with results obtained by other investigators. The permeability was equal in both directions, and thus the capillary membrane seems to function as a symmetrical membrane. Using an area of 5 000 cm²/100 g, such permeability is more realistic in the plasma flow range used gives P_d values around $1.5 \cdot 10^{-6}$ cm/s. An effective pore area is calculated to constitute 1/30 000 of the capillary surface area. Calculation of effects of distribution in the muscle tissue gave intravascular plasma volumes of 1.8 to 2.0 ml/100 g, an extravascular volume of 12.4 and 15.2 ml/100 g and final monoexponential component constituting compartment of 5.4 and 7.0 ml/100 g from residue and venous curves, respectively. The last mentioned compartment constituted nearly 50 per cent of the extravascular space, and it is suggested, that it is located inside the sarcolemma reticulus, which anatomically constitutes about 50 per cent of the interstitial space. The total area of contact between the longitudinal and the transversal tubules in this sarcolemma, which is the membrane of the lateral sacculus, is extended to about 6 times the capillary surface area at plasma flow 4.15 ml/100 g min, which gives permeability about 60 times lower for this membrane compared to the capillary membrane.

Measurement of capillary permeability has been attempted by various techniques (Pappenheimer, Renkin and Borrero 1951, Chinard, Vosburg and Enns 1955, Crone 1963). Some of these techniques have been criticized with respect to their theoretical basis (Crone 1970). The present article presents an alternative technique which is based on intra-arterial bolus injection of a γ -emitting indicator and residue or venous detection (Sejnsen 1970, preliminary report). The injected indicator serves as its own intravascular reference and permeability data are obtained by kinetic analysis of the recorded response function. In the present article a more detailed treatment of the theoretical basis of the method has been performed.

- DAY M D, J W MCCURBIN and I H PAGE Limited hypertensive effect of infusion of angiotensin. *Amer J Physiol.* 1965 209 264-268.
- FREEMAN, R. H. and J D DAVIS, Control of renin secretion. In J GENEST, E. KORW and O. KOTM *Hypertension* McGraw-Hill, New York 1977 p. 210-239
- KRESIAK and J JANKU The development of aggressive behaviour in mice by isolation. In E. GARATTI and E. B. SGOO, *Aggressive Behaviour* Amsterdam 1969 70-76.
- LAGERPETZ, K. M J Aggression in mice 1969 *Ibidem* 77-85
- MALLINO C. and K. POULSEN, A direct radioimmunoassay for plasma renin in mice and its evaluation *Biochem. biophys. Acta.* (Amst.) 1977 a. 491 532-541
- MALLINO C. and K. POULSEN, Direct measurement of high molecular weight forms of renin in plasma *Biochem. biophys. Acta* 1977 b. 491 542-550.
- MENZIE, J. W., A. M. MICHELAKIS and H. YOSHIDA, Sympathetic nervous system and renin release from submaxillary glands and kidneys. *Amer J Physiol.* 1974. 227 1281-1284.
- PETTINOER, W. A., K. TANAKA, K. KEETAN, W. B. CAMPBELL and S. N. BROOKS, Renin release, an arch of anaesthesia. *Proc Soc exp Biol Med.* 1975 148. 625-630.
- POULSEN, K. and J. JOERGENSEN, An easy radioimmunological microassay of renin activity cooceptor and substrate in human and animal plasma and tissues based on angiotensin I trapping by antibody *J clin Endocrinol. Metab.* 1974 39 816-825
- VALZELLI, L., Aggressive behaviour induced by isolation. In S. GARATTI and E. B. SGOO, *Aggressive Behaviour* Amsterdam 1969 167-171

5 Isoefficiency counting, *i.e.* equal counting efficiency for individual fractions of the indicator dose throughout the passage through the vessel and the tissue area seen by the detector but there is no demand of equal counting efficiency from one tissue region to other.

6 Recirculation is eliminated, negligible or known.

7 The height of the registered curve is a relative measure of the total amount of indicator injected. \Rightarrow all indicator molecules must be present in the field seen by the detector at a certain time.

8 The early part of the curve for the extracted fraction must be deducible from the later part of the curve, *i.e.* the washout of the extracted fraction can be simulated by some mathematical function and extrapolated retrogradely to peak time yielding a relative measure of the extracted amount of indicator as the numerical value of the retroplotted curve at peak time. This time is the time where the total amount of indicator is present in the counting field and therefore also the time where total amounts of the two fractions are present. The retroplation can be performed from a time where a vascular transit after a bolus injection of a vascular indicator has come to an end for all practical purposes.

If these assumptions are fulfilled the extracted fraction, $E(\text{res})$, can be calculated from the residue detection curve as the height of the curve representing the transit of the extracted fraction, $H(\text{ev})$, obtained by retroplation to peak time, divided by the height of the registered curve, $H(\text{total})$:

$$E(\text{res}) = \frac{H(\text{total})}{H(\text{ev})} \quad (1)$$

The curve of the transmitted fraction, T , obtained by numerical subtraction of the extrapolated curve from the detected curve allows calculation of the mean transit time of this fraction and if the vascular volume and the hematocrit are known plasma and blood flow can be computed. Experimentally it is possible to validate the performed curve resolution by comparison of the results to plasma flow calculated from directly measured venous outflow and the curve configuration to that of an intravascular indicator.

Transition from tissue to blood

In this connection the extracted fraction, E , means the fraction exchanged from tissue to blood related to that obtainable at equilibrium over the capillary membrane.

When followed to infinity the residue curve contains information of indicator permeation in the tissue to blood direction. To obtain this information the nine assumptions mentioned above must be fulfilled together with the condition

9 That the total area under the curve from time zero to infinity can be estimated.

The mean transit time, \bar{t} , for an indicator through a system is the mean value of transit times for the indicator particles.

$$\bar{t} = \frac{t_1 + t_2 + t_3 + \dots + t_n}{n} \quad (2)$$

where $t_1, t_2, t_3, \dots, t_n$ denote the transit times of each particle, and n is the total number of particles.

To evaluate this method experimentally it was used simultaneously with the indicator dilution method which employs a venous sampling technique and simultaneous bolus injection of a permeating test substance and an intravascular reference substance (Chinard *et al.* 1963; Crone 1963).

The method presented uses uptake as well as washout principles and measures the microcapillary extraction fraction in both directions over the membrane in the same experiment. The venous outflow curve, which is the differentiated analogue of the residue curve, allows use of β -emitting and non radioactive indicators. The four calculations of the extraction fraction represents four independent estimates.

From a tracer kinetic point of view the presented principles attempt to get separate information from subsystems inside the main system.

Theoretical considerations

Residue detection method

Extraction from blood to tissue

When an indicator which is able to permeate the capillary membrane is injected intravascularly as a bolus the molecules are divided into two fractions according to their movements during the passage of an organ or tissue. One fraction, called the extracted fraction, consists of molecules which leave and reenter the blood one or more times during the transit. The remaining molecules, denoted the transmitted fraction, constitute the complementary fraction which stays intravascularly during the transit. Registration of the indicator residue is possible by external counting when a γ -emitting indicator is used. The rapid transit of the transmitted fraction is very fast as compared to the transit of the extracted fraction which also distributes in the larger extravascular space. This space functions in the initial phase as a sink for the indicator when this is a foreign substance. For these reasons the residue curve has a steep early downslope the formation of which is dominated by the fast vascular transit of the transmitted fraction through the relatively small intravascular volume, followed by a curve part with decreasing rate constant which is dominated by the slow transit of the extracted fraction via the relatively greater extravascular space.

The great difference in mean transit times for the two fractions of the indicator makes it possible to resolve with good approximation the recorded residue curve in the individual transit curves for the transmitted and the extracted fraction.

The assumptions of the kinetic analysis are

1. Conservation of matter *i.e.* the indicator substance in question is maintained or altered inside the system.
2. Stationarity of the system, *i.e.*, the system is assumed to be in steady state with respect to blood flow, distribution of transit times and volumes of distribution, and permeability.
3. Linearity of the system, *i.e.* linearity (proportionality) between input and output for different sizes of input.
4. Equivalent entry *i.e.*, the indicator particles are completely mixed with the inflowing blood (plasma) at the entrance to the system.

2.5 Isoefficiency counting, *i.e.* equal counting efficiency for individual fractions of the indicator dose throughout the passage through the vessel and the tissue area seen by the detector but there is no demand of equal counting efficiency from one tissue region to other

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The curve of the transmitted fraction, T obtained by numerical subtraction of the extrapolated curve from the detected curve allows calculation of the mean transit time of this fraction and if the vascular volume and the hematocrit are known plasma and blood flow can be computed. Experimentally it is possible to validate the performed curve resolution by comparison of the results of plasma flow calculated from directly measured venous outflow and the curve configuration to that of an intravascular indicator

Extraction from tissue to blood

In this connection the extracted fraction, E , means the fraction exchanged from tissue to blood related to that obtainable at equilibrium over the capillary membrane.

When followed to infinity the residue curve contains information of indicator permeation in the tissue to blood direction. To obtain this information the nine assumptions mentioned above must be fulfilled together with the condition

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The mean transit time, \bar{t} , for an indicator through a system is the mean value of the times for the indicator particles.

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TRANSITION FROM TISSUE TO BLOOD

In this connection the extracted fraction, E , means the fraction exchanged from tissue to blood related so that obtainable at equilibrium over the capillary membrane.

When followed to infinity the residue curve contains information of indicator permeation in the tissue to blood direction. To obtain this information the nine assumptions mentioned above must be fulfilled together with the condition,

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where t_1, t_2, \dots, t_n denote the transit times of each particle, and n is the total number of particles.

For any system, or subsystem which is only traversed once by the particles the mean transit time can be determined as the area, A , divided by the height, H , of the residue curve (Zierler 1965)

$$\bar{t} = \frac{A}{H}$$

The mean sojourn time, θ is the mean occupancy time of indicator particles within system or subsystem placed inside the main system. For the total system $\bar{t} = \theta$ but for subsystem θ can be greater than \bar{t} if the indicator particles traverses the subsystem more than once.

As determined by residue detection the equation for the mean sojourn time, θ in a system or subsystem is analogous to equation 3

$$\theta = \frac{A}{H}$$

where A and H is area and height of the curve for system or subsystem.

In the present experiments the mean transit time of the indicator for the whole system $\bar{t}(\text{total})$, is the sum of the mean transit time through the subsystem of the vascular volume of distribution, $\bar{t}(\text{iv})$, and the mean sojourn time in the subsystem, the extravascular volume of distribution, $\theta(\text{ev})$, times the extracted fraction E . All indicator particles traverse vascular volume of distribution only once due to domination of convective transport in subsystem, but in the extravascular volume of distribution there is a possibility for more than one traversal of some indicator particles. The total amount of indicator traverses vascular volume of distribution but only the fraction E of this permeates into the extravascular volume of distribution. This leads to the following equation

$$I \bar{t}(\text{total}) = I \bar{t}(\text{iv}) + E \theta(\text{ev})$$

where I is the total amount of indicator given as a fraction $(I = T + E)$

If the curve representing the transit of the extracted fraction, in the following called extravascular curve, is to be described by more than one exponential function, E is not constant during the washout process. But it is possible to obtain an average value \bar{E} denoted $\bar{E}(\text{res})$ for the whole washout process from tissue to blood measured by residue detection. In the following expression equation (5) is solved for E , and $\bar{E}(\text{res})$ is introduced

$$\bar{E}(\text{res}) = \frac{\bar{t}(\text{total}) - \bar{t}(\text{iv})}{\theta(\text{ev})}$$

Venous sampling method

Extraction from blood to tissue

From the venous outflow function it is possible to calculate the extraction from blood to tissue, by a curve extrapolation analogous to that described for the residue detection technique. It is by the venous sampling technique also possible to use β -emitting and non-radioactive indicators.

Fulfillment of assumptions number 1, 2, 3, 4, 6, and 9 are also needed for the venous sampling technique. As area under a venous curve represents the same as height on the residue curve, the extraction fraction, $E(\text{vein})$, can be calculated in analogy with equation (1):

$$E(\text{vein}) = \frac{A(\text{ev})}{A(\text{total})} \quad (7)$$

where $A(\text{ev})$ and $A(\text{total})$ are the areas under the extravascular and the total venous concentration curves, respectively

Extraction from tissue to blood

On the basis of assumptions number 1, 2, 3, 4, 6, and 9 and analogous to the transformation of area on the venous curve to height on the residue curve and time weighted area on the venous curve to area on the residue curve, the venous sampling technique allows calculation of the average extraction, $\bar{E}(\text{vein})$, also for the transcapillary passage in the direction from tissue to blood. The time weighted area is defined as the sum of the products of time and counting activity (or concentration) for each sample. Transforming the ratio area over weight for the residue curve, $A(\text{res})/H(\text{res})$, to the ratio time weighted area, A_w over area, A , for the venous concentration curve, $A_w(\text{vein})/A(\text{vein})$, in calculation of the mean transit times and the mean sojourn time then the right side of the equation becomes the same as in equation (6)

$$\bar{E}(\text{vein}) = \frac{\bar{t}(\text{total}) - \bar{t}(\text{rv})}{\bar{t}(\text{ev})} \quad (8)$$

For estimation of mean transit times and sojourn time the catheter effect must be corrected for in the venous sampling technique. This can be performed by simple subtraction of the mean transit time for the catheter from all three terms on the right side in equation (8). This same minus and plus the same value in the nominator and it cancels out. Thus only the denominator has to be corrected in this calculation. If the volume of the catheter and the outflow from the system are measured the mean transit time for the catheter $\bar{t}(\text{cath})$, can be calculated (Zierler 1965).

$$\bar{t}(\text{cath}) = \frac{V(\text{cath})}{F} \quad (9)$$

where, $\bar{t}(\text{cath})$, is the mean transit time of the catheter $V(\text{cath})$, the catheter volume, and F the blood flow. In analogy with equation (9) blood flow through the vascular volume can be calculated from the mean transit time of the intravascular transit of an indicator and the vascular distribution volume. This general equation makes it also possible to calculate volumes of distribution from mean transit time and flow. For the subsystem the extravascular volume of distribution the \bar{t} is used instead of \bar{t} in the calculation.

The final monoexponential component can be taken to represent washout from a well mixed biological compartment.

The indicator diffusion method (Chinard *et al.* 1955 and Crone 1963) employed in this comparative study has the following assumptions number 1, 2, 3, 4, and 6 mentioned above and in addition.

For any system, or subsystem, which is only traversed once by the particles the transit time can be determined as the area, A , divided by the height, H , of the residue curve (Zierler 1965)

$$\bar{t} = \frac{A}{H}$$

The mean sojourn time, θ , is the mean occupancy time of indicator particles within system or subsystem placed inside the main system. For the total system $\bar{t} = \theta$ but for subsystem θ can be greater than \bar{t} if the indicator particles traverses the subsystem more than once.

As determined by residue detection the equation for the mean sojourn time, θ , in a system or subsystem is analogous to equation 3

$$\theta = \frac{A}{H}$$

where A and H is area and height of the curve for system or subsystem.

In the present experiments the mean transit time of the indicator for the whole system $\bar{t}(\text{total})$ is the sum of the mean transit time through the subsystem of the vascular volume of distribution, $\bar{t}(\text{iv})$ and the mean sojourn time in the subsystem, the extravascular volume of distribution, $\theta(\text{ev})$, times the extracted fraction E . All indicator particles traverse the vascular volume of distribution only once due to domination of convective transport in the subsystem, but in the extravascular volume of distribution there is a possibility for more than one traversal of some indicator particles. The total amount of indicator traverses the vascular volume of distribution but only the fraction E of this permeates into the extravascular volume of distribution. This leads to the following equation

$$1 \bar{t}(\text{total}) = 1 \bar{t}(\text{iv}) + E \theta(\text{ev})$$

where 1 is the total amount of indicator given as a fraction ($1 = T + E$)

If the curve representing the transit of the extracted fraction, in the following called extravascular curve, is to be described by more than one exponential function, E is not constant during the washout process. But it is possible to obtain an average value \bar{E} denoted $\bar{E}(\text{res})$ for the whole washout process from tissue to blood measured by residue detection. In the following expression equation (5) is solved for E , and $\bar{E}(\text{res})$ is introduced

$$\bar{E}(\text{res}) = \frac{\bar{t}(\text{total}) - \bar{t}(\text{iv})}{\theta(\text{ev})}$$

Venous sampling method

Extraction from blood to tissue

From the venous outflow function it is possible to calculate the extraction from blood to tissue, by a curve extrapolation analogous to that described for the residue detection technique. It is by the venous sampling technique also possible to use β -emitting and non-radioactive indicators.

corrections for values of blood of plasma are performed. The Chromium-51 or Iodine-131 activity spectra and strips were counted in a well-type scintillation detector (NaI (TD 2 inches diameter) used around the maximum γ -peak of the respective radioisotopes. Counting as continued until about 10 counts or more had been recorded.

The blood pressure was continuously recorded by a Statham (p 23 AA) transducer with saline filled and placed in the contralateral femoral artery. Also injections of Chromium-51-EDTA (ethylene-diamine-tetra-acetic-acid) or Iodine-131-thalamus were performed to serum albumin started in isotonic saline were performed while about 1 second. Volume of injection was 0.1 ml. Immediately after the injection the contents in the catheter as withdrawn by the injection syringe.

Measurements and calculations were performed in accordance with the procedures described above.

The peak heights of the measured curves were calculated as an average of the 4-6 highest consecutive count figures.

Extrapolation to peak time of the registered curve, as measured after the end of the time interval of the color transit, was performed either by analyzing this curve part by the sum of three exponentials or monoexponential curve fitting of the early part of the curve from 1 to 3 minutes after the bolus injection.

The last mentioned procedure was performed as a compromise between minimum effect of the aging of the curve and reasonable number of observations on which regression analysis could be formed. The interval before peak time the extrapolation was performed by multiplying the measured area by the ratio between the height of the extrapolated curve at peak time and the peak value of the measured curve. For the peak plasma the same calculation procedure was used for this interval. The tribution of the measured values on the upstroke part of the curve was done in order to calculate the area under the curve. For the same reason it was necessary to extrapolate the curve to infinity. This was performed by monoexponential function with the rate constant of the later part of the registered curve (Lassen and Sejersen 1971). This extrapolation procedure was used both on residue and venous outflow curves. The area under the tail part of the residue curve, $A(t_{\text{tail}}, \text{res})$, extrapolated to infinity was calculated as the half-time ($T_{1/2}$) of the monoexponential down slope of the later part of the curve by the formula

$$A(t_{\text{tail}}, \text{res}) = H(t_{\text{tail}}) \frac{T_{1/2}}{\ln 2} \quad (10)$$

where $H(t_{\text{tail}})$ is the height of the tail part at the end of the experiment, and $\ln 2$ is the natural logarithm 2.

The area under the venous curve $A(t_{\text{tail}}, \text{ven})$, as obtained in another way:

$$A(t_{\text{tail}}, \text{ven}) = H(t_{\text{tail}}) \frac{T_{1/2}}{\ln 2} \quad (11)$$

The time weighted area under the venous curve, $Aw(t_{\text{tail}}, \text{ven})$, was calculated by the formula:

$$Aw(t_{\text{tail}}, \text{ven}) = H(t_{\text{tail}}) \frac{T_{1/2}}{\ln 2} \left(\frac{T_{1/2}}{\ln 2} \right) \quad (12)$$

where t_{tail} is the time at the end of the experiment.

Analysis with equation (9) plasmaflow was computed using the extravascular mean transit time, $\bar{t}_e(v)$, obtained by retroprojection and subtraction, and value for the vascular plasma volume obtained in previous experiments. Furthermore, the plasma volume was calculated from the $\bar{t}_e(v)$ measured for the measurement curves using the same equation and the directly measured blood flow and the hematocrit value.

In accordance with the single injection, venous sampling technique (Clineard *et al.* 1953, Crowe 1963), calculation of the capillary extraction fraction (E) was performed by the formula:

$$E = 1 - \frac{\text{relative concentration of test substance in sample}}{\text{relative concentration of intravascular indicator in sample}} \quad (13)$$

The concentrations in the samples are here given relative to the concentration in the injectate. Calculation of an average extraction value was performed by taking the ratio between the area under the test substance curve with the time where the reference curve was reduced to 40 per cent of maximum and the area under the curve of the reference substance in the same time interval. This average ratio was then inserted in equation (13) instead of the ratio of the relative concentrations of the two substances in one sample.

10 The reference molecules must be representative for the vascular transit of the molecules, *i.e.* equal Taylor diffusion conditions for the two molecules (Lassen and Ch 1970)

11 Back diffusion of indicator from the extravascular space to the blood must be negligible. In the initial phase, *i.e.* the extravascular space must function as a sink for the indicator when this is a foreign substance

Experimental

Isolated autoperfused gastrocnemius preparation

The experiments were performed on 7 cats weighing from 3.0 to 4.5 kg. After ether induction chloralose (70 mg/kg) was given intravenously. Tracheostomy was performed, and the rectal temperature of the cat was kept at 37°C by heating lamps controlled by a thermostat. Heparin (5 mg/kg) was given intravenously.

The skin of the right leg down to the paw was removed by thermocautery. The tendo calcaneus was close to calcaneus, and the gastrocnemius muscle was isolated by blunt dissection. The thigh muscle was divided just proximal to the knee joint, and crus was removed by disarticulation. The cavity of the femoral bone was plugged by cotton soaked in vaseline and the periosteal membrane was removed. The femoral artery and vein were isolated at the lower 2/3 of the thigh, and all side branches were ligated, except one distal branch of the artery in which a fine polyethylene catheter was inserted until the tip was at the level of the wall of the femoral artery. The femoral vein was cut and cannulated with a 1.5 cm long polyethylene tube (i.d. 1.5 mm) connected to a 16 cm long glass tube (i.d. 2.8 mm) used as venous catheter and placed in level with the muscle. The muscle was wrapped in moist gauze and covered by a polyethylene sheet which was kept in place during the preparation and throughout the experiment. The temperature of the muscle was kept at 37°C by a heating lamp. The muscle was suspended on a stand, with a length of the muscle equal to the normal resting length. Different blood flow levels, both fairly high and low, in each experiment, were obtained by electrical stimulation of the isolated sciatic nerve by silver electrodes using a S4 Grass stimulator. The frequency was from 1 per 3 seconds to 1 per second, the charge 75 μ C, the duration of the square pulses was 1 msec. Blood flow was measured directly as venous outflow in a graduated cylinder and a stop watch, several times before and during each experiment. Hematocrit values were measured in these blood samples. To avoid recirculation the venous blood from the isolated gastrocnemius muscle was wasted and the cat was given donor blood from 2 to 5 cats. This amount of blood was sufficient to give the experiments a duration of about 2 hours.

The carefully shielded scintillation detector a NaI(Tl) crystal with diameter of 1 1/4 inch, 15 cm long, was positioned 10 cm medially to the muscle and collimated to see only muscle tissue properly. The pulses from the scintillation detector were fed into a scaler printer (Med tronac, Denmark) which was adjusted to print the γ -emission around the maximum energy peaks of Chromium-51 or Iodine-131. The count rates were printed out every half or one second in the initial 60 seconds period and every minute in the following two hours.

In order to reduce the background activity the whole muscle preparation was surrounded by lead which resulted in a stable background activity of about 2 counts per second.

At the end of each experiment the femoral artery and vein were clamped and the perfusion stopped. The counting level (a) was registered in 10 intervals from 15 to 30 min. The polyethylene and gauze covering the muscle were removed and the muscle surface was gently washed with soap and water to remove the small amount of tracer deposited on the surface. In another 15 to 30 minutes period the counting level (b) was measured under the same conditions. A third counting period (c) of the same length followed after separate removal of the muscle. Correction for background activity and the activity deposited on the surface of the muscle and the gauze was performed as a subtraction of the activity levels a-(b-c) from the corrected curve with a final activity equal to that of the muscle tissue separately.

After the experiment the weights of both gastrocnemius muscles were measured. A weight difference was taken as weight change of the test muscle during the experiment.

Venous sampling was performed every 2 seconds during the initial 60 seconds and every fifth second during the following 1 to 2 hours. The samples (0.1 to 0.5 ml) were taken in small glass vials. Plasma samples were obtained by centrifugation.

The T_{max} concentrations in the arterial and venous plasma samples were estimated by a Beckman spectrophotometer at 620 nm.

1. Curve measured after bolus injection of Cr 51-EDTA and re-detection. Registration of initial 3 min with 3-exponential and monoexponential retro-spection to peak time of the extracellular part. The triangles show the intravascular curve obtained by subtraction of the areas for the 3-exponential curve from the registered curve.

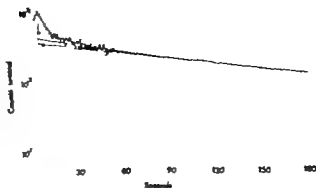


Table I lists experimental data from the 7 expts. Muscle weight, the weight excess of the muscle compared to the contralateral muscle, and hematocrit values in per cent. Furthermore, this table shows plasma flow calculated from the directly measured venous outflow in initial phase and as average value $\bar{x} \pm SE$ for the whole experiment, and plasma flows obtained from the intravascular transit curve obtained by residue detection and venous plugging. In these calculations a value for the intravascular volume of distribution of 3.0 cm³ of the tissue volume was used (see supplementary experiments denoted 4), and \bar{x} . Average values $\bar{x} \pm SE$ for experiment number 1 to 6 or 3 to 6 are listed below for four last mentioned parameters.

Table II presents the extraction fraction as estimated by 3 independent calculations in first 3 experiments and by 5 in expt. number 4 to 7. No significant differences were found between the extraction values obtained by the five methods. Cr 51-EDTA was used

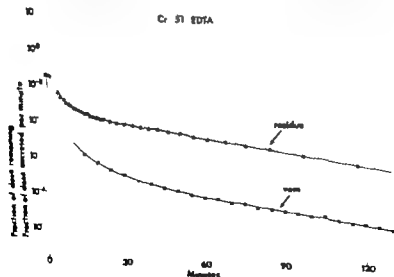


Fig. 2. Curves measured after bolus injection of Cr 51-EDTA with residue and venous outflow detection scaled to the height 1 for the residue curve and the area 1 for the venous curve.

Calculation of the capillary diffusion capacity (CDC) was performed from the equation.

$$CDC = -f_{pl} \cdot 0.89 \ln(1 - E) \text{ (ml/100 g min)}$$

where f_{pl} is flow of plasma per 100 g tissue, \ln is the natural logarithm, and 0.89 is a factor correcting the concentration differences in plasma and interstitial fluid at equilibrium (Lassen and Trap-Jensen 1971) taking into account both the difference in water space in the two phases and the effect of the transmembrane potential difference for negatively charged, monovalent ions.

Supplementary experiment

Special experiments were performed to study 1) the input function, 2) the fastest transit through the system measured by the residue detection or venous sampling without catheter, 3) the response curve for venous catheter, and 4) the vascular volume of distribution in the muscle.

1) The input function to the autoperfused gastrocnemius preparation was studied by cutting the femoral artery at the outer zone of the external counting field. After cutting the artery the blood flow was stopped by a screw clamp and kept constant at levels used in the other experiments. The bolus injection technique described above was used for a bolus of Cr 51 labelled erythrocytes. When the blood was leaving the artery it passed the counting field falling through the air and it was collected in a glass vial situated outside counting field. By this technique the output function from the artery to the counting field was obtained, i.e. the input curve to the muscle.

2) The fastest transit through the system was studied by cutting the femoral vein at the outer zone of external counting field where sampling was performed directly in small glass vials. Cr 51 labelled erythrocytes were used as indicator. In this series of experiments as in the following the bolus injection technique and blood flow at the levels of the main series of experiments were used.

3) Bolus response curves for the catheter alone were obtained during perfusion from a blood reservoir by hydrostatic pressure force. In these experiments the temperature of the blood in the reservoir leading to the catheter and the catheter was kept at 37°C. A bolus of Cr 51 labelled erythrocytes was injected directly into the initial part of the inflow end of the venous sampling catheter. Outflow samples were taken as in the main experiments.

4) Vascular volume of distribution was determined using the intravascular tracers T_{125I} -albumin and Cr 51 labelled erythrocytes. The mean transit time of these tracers multiplied by the directly measured venous outflow of blood yielded the intravascular volume for the tracers in analogy with equation (1) and by using the hematocrit value the intravascular plasma volume was computed.

Results

Chromium-51 EDTA was used as test substance in six experiments and Iodine 131-labelled albumin in one. T_{125I} -albumin was used as an intravascular indicator in all experiments. Representative residue curves plotted in semilogarithmic scale are shown in Fig. 1 and 2 with different time axis. In Fig. 1 is shown the initial 3 min of the residue curve with a fitting of the curve after the first minute by the sum of three exponentials and extrapolation back to peak height as described above. The sum of three exponentials fitted the curves very well in the time interval 1 to 130 minutes after the bolus injection as seen in Fig. 2, where the line drawn through the dots is the sum of three exponentials. Also a monoexponential retroprojection with a curve fitting from 60 to 180 seconds is shown in Fig. 1 (the lower line). In Fig. 2 the residue curve is scaled to peak height equal to one and the simultaneously measured venous curve also presented in this figure is scaled to area under the curve equal to one.

The bolus injection venous sampling method using vascular reference indicator is presented in Fig. 3 and 4. The concentrations of test and reference indicator in samples relative to the concentration in injectate are plotted linearly as a function of time in Fig. 3. For the same experiment the extraction values calculated from equation (13) are plotted versus time in Fig. 4.

1. Curv. measured after bolus injection of Cr 51 EDTA and re-
sidual detection. Registration of
initial 3 min. with 3-exponential
and monoexponential retro-
trace to peak time of the extracur-
vular curve part. The triangles
are the intravascular curve
obtained by subtraction of the
residuals for the 3-exponential curve
from the registered curve.

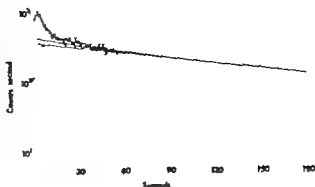


Table I lists experimental data from the 7 expts. Muscle weight, the weight excess of the muscle compared to the contralateral muscle, and hematocrit values in per cent. Further are, this table shows plasma flows calculated from the directly measured venous outflow the initial phase and as average value $\bar{x} \pm SE$ for the whole experiment, and plasma flows calculated from the intravascular transit curve obtained by residual detection and venous sampling. In these calculations a value for the intravascular volume of distribution of 3.0 per cent of the tissue volume was used (see supplementary experiments denoted 4), and (low). Average values $\bar{x} \pm SE$ for experiment number 1 to 6 or 3 to 6 are listed below for the four last mentioned parameters.

Table II presents the extraction fraction as estimated by 3 independent calculations in the first 3 experiments and by 5 in expt. number 4 to 7. No significant differences were found between the extraction values obtained by the five methods. Cr 51-EDTA was used

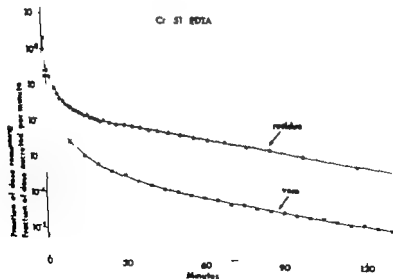


Fig. 2. Curves measured after bolus injection of Cr-51-EDTA with residual and venous outflow detection scaled to the height 1 for the residual curve and the area 1 for the venous curve.

Calculation of the capillary diffusion capacity (CDC) was performed from the equation:

$$\text{CDC} = -f_p \cdot 0.89 \ln(1 - E) (\text{ml}/100 \text{ g min})$$

where f_p is flow of plasma per 100 g tissue. \ln is the natural logarithm, and 0.89 is a factor concerning the concentration differences in plasma and interstitial fluid at equilibrium (Lassen and Trap-Jensen 1967) taking into account both the difference in water space in the two phases and the effect of the transmembrane potential difference for negatively charged, monovalent ions.

Supplementary experiments

Special experiments were performed to study 1) the input function, 2) the fastest transit through the system measured by the residue detection or venous sampling without catheter, 3) the response curve in venous catheter, and 4) the vascular volume of distribution in the muscle.

1) The input function to the autoperfused gastrocnemius preparation was studied by cutting the femoral artery at the outer zone of the external counting field. After cutting the artery the blood flow was stopped by a screw clamp and kept constant at levels used in the other experiments. The bolus injection method described above was used for a bolus of Cr 51 labelled erythrocytes. When the blood was leaving the artery it passed the counting field falling through the air and it was collected in a glass vial situated outside the counting field. By this technique the output function from the artery to the counting field is obtained, i.e. the input curve to the muscle.

2) The fastest transit through the system was studied by cutting the femoral vein at the outer zone of the external counting field where sampling was performed directly in small glass vials. Cr 51 labelled erythrocytes were used as indicator. In this series of experiments as in the following the bolus injection method and blood flow at the levels of the main series of experiments were used.

3) Bolus response curves for the catheter loop were obtained during perfusion from a blood reservoir by hydrostatic pressure force. In these experiments the temperature of the blood in the reservoir, the tube leading to the catheter and the catheter was kept at 37°C. A bolus of Cr 51 labelled erythrocytes was injected directly into the initial part of the inflow end of the venous sampling catheter. Outflow samples taken as in the main experiments.

4) Vascular volume of distribution was determined using the intravascular tracers T_{199} -albumin and Cr-51 labelled erythrocytes. The mean transit time of these tracers multiplied by the directly measured venous outflow of blood yielded the intravascular volume for the tracers in analogy with equation (1) and by using the hematocrit value the intravascular plasma volume was computed.

Results

Chromium-51 EDTA was used as test substance in six experiments and Iodine-131-albumin in one. T_{199} -albumin was used as an intravascular indicator in all experiments. Representative residue curves plotted in semilogarithmic scale are shown in Fig. 1 and 2 with different time axis. In Fig. 1 is shown the initial 3 min of the residue curve with a fitting of the curve after the first minute by the sum of three exponentials and extrapolation back to peak height as described above. The sum of three exponentials fitted the curves very well in the time interval 1 to 130 minutes after the bolus injection as seen in Fig. 2, where the line drawn through the dots is the sum of three exponentials. Also a monoexponential retroprojection with a curve fitting from 60 to 180 seconds is shown in Fig. 1 (the lower line). In Fig. 2 the residue curve is scaled to peak height equal to one and the simultaneously measured venous curve also presented in this figure is scaled to area under the curve equal to one.

The bolus injection venous sampling method using vascular reference indicator is presented in Fig. 3 and 4. The concentrations of test and reference indicator in samples relative to concentration in injectate are plotted linearly as a function of time in Fig. 3. For the same experiment the extraction values calculated from equation (13) are plotted versus time in Fig. 4.

1. Can. measured after bolus
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are the intravascular curve
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area for the 3-exponential curve
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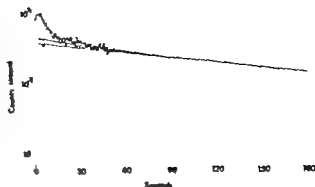


Table I lists experimental data from the 7 expts. Muscle weight, the weight excess of the muscle compared to the contralateral muscle, and hematocrit values in per cent. Furthermore, this table shows plasma flows calculated from the directly measured venous outflow in the initial phase and as average values $\bar{x} \pm SE$ for the whole experiment, and plasma flows calculated from the intravascular transit curve obtained by residue detection and venous sampling. In these calculations a value for the intravascular volume of distribution of 3.0 per cent of the tissue volume was used (see supplementary experiments denoted 4), and (below) Average values $\bar{x} \pm SE$ for experiment number 1 to 6 or 3 to 6 are listed below for the four last mentioned parameters.

Table II presents the extraction fraction as estimated by 3 independent calculations in the first 3 experiments and by 5 in expt. number 4 to 7. No significant differences were found between the extraction values obtained by the five methods. Cr 51 EDTA was used

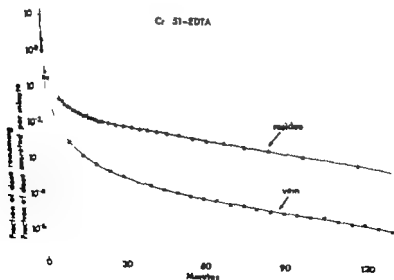


Fig. 2. Curves measured after bolus injection of Cr-51-EDTA with residue and venous outflow detection related to the height 1 for the residue curve and the area 1 for the venous curve.

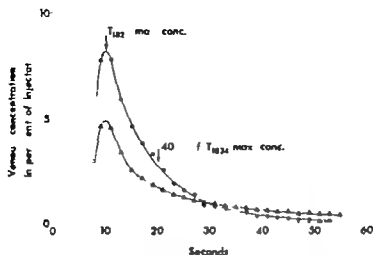


Fig. 3. Washout curves after injection of T_{182} -albumin and Cr-51-EDTA registered by venous sampling. Dots and triangles notes the T_{182} -albumin and Cr-51-EDTA, also given in per cent of the injectate.

as indicator in expt. number 1 to 6 and I 131 thalamate in expt. number 7. The capillary diffusion capacity CDC was calculated from directly measured plasma flows, and values $\bar{x} \pm SE$ for expt. number 1 to 6 and 4 to 6 are listed below. Furthermore, CDC calculated from plasma flows obtained by curve resolution for the four methods are given with average values $\pm SE$ for expt. number 1 to 6 and 4 to 6. No significant differences were found between the CDC values obtained by the five independent methods.

Monoexponential retroprojection to peak time of the extravascular transit curve in the interval from 60 to 180 s was found to underestimate the extraction with 11.8 per cent on average compared to the 3 exponential curve resolution. Also the estimate of the extravascular mean transit time was different with the two extrapolations. Monoexponential analysis gave an overestimation of this value by 9.5 per cent which in part counterbalanced the underestimation of the E value at the calculation of the CDC value. The combined effect on the CDC value is on average a 3.2 per cent underestimation by the monoexponential extrapolation as compared to the three exponential extrapolation.

The extravascular curves showed a bended shape with on average a factor of 12.8 between the rate constant of the beginning and the final curve part.

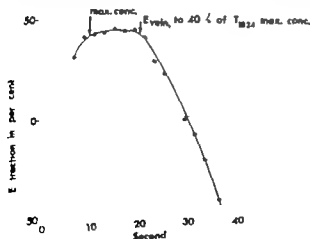


Fig. 4. Extravascular curves calculated by values of T_{182} -albumin and Cr-51-EDTA for each sample.

TABLE II. Muscle weight, edema, hematocrit, initial and average plasma flows. For expts. no. 1 to 6 and 4 to 6, average \pm SE are given below

Expt. no.	Muscle weight (g)	Muscle edema (per cent)	Hematocrit (per cent)	Plasma flow initial phase (ml/100 g min)		Plasma flow average values (ml/100 g min)	
				Calculated from directly measured blood flow	Calculated from residue curve	Calculated from venous curve	Calculated from directly measured blood flow SE
1	19.6	7.1	37.4	15.8	19.4		0.29
2	28.7	12.4	34.6	25.5	24.5		0.45
3	11.6	1.6	36.6	12.1	17.8		0.04
4	42.4	0.2	38.4	11.9	9.8	9.7	0.27
5	29.5	10.1	34.8	15.1	17.5	16.3	0.23
6	18.8	4.3	34.0	11.4	11.8	9.8	0.07
7	27.8	17.3	33.5	18.4	17.4	22.9	0.20
SE				6 14.7 ± 1.8	6 16.8 ± 2.2	3 1.0 ± 2.2	6 13.9 ± 2.0

Data from expt. number 4 to 7 have been presented in a previous article concerning nonexponential extrapolation of the final slowest part of washout curves (Lassen and Green 1971). The corresponding numbers of the expts. in that article are 1, 3, 5 and 7. In each of these expts. there was a close agreement between the rate constants calculated from the final curve parts of the residue and venous curves. Three experiments from the quoted article were excluded from the material presented in this article: two (no. 4 and 6) due to lack of agreement between these two final rate constants within the single expts., and one (no. 4) due to a delayed bolus input.

Table III shows the volumes of distribution for the indicators as calculated from the residue and venous curves as the total system, in the intravascular part, in the interstitial part and in the part defined by the monoexponential tail of the curve. Average values \pm SE for expts. number 1 to 7 and 4 to 7 are listed below.

Calculation of the intravascular plasma volume from the T_{intra} -albumin curves with correction for the effect of the catheter gave in average \pm SE: 1.9 ± 0.1 ml/100 g of tissue. This is in agreement with the results obtained by kinetic analysis.

The results of the supplementary experiments were the following:

1) The input function to the muscle had a duration of about 1 s during which 99 per cent of the indicator had arrived (2 expts.) at plasma flows used in the main series. The input function can also be evaluated from the increasing part of the residue curves.

2) The shortest transit times determined by venous sampling were from 3 to 6 s at plasma flow values as those in the main series (4 expts.). The venous curves for intravascular indicators could be simulated by the sum of two exponentials with a factor of about 2 to 5 between the rate constants of the fast and the slow component. The interception with the ordinate were about 88 per cent of the venous curve height for the fast and 5 per cent for the slow component.

TABLE II. Capillary extraction fraction measured simultaneously by 5 independent methods with corresponding values for capillary diffusion capacity. For expt. no. 1 to 6 or 4 to 6, average \pm are given below. The CDC values are given in ml/100 g min.

Expt no	Indicator	E ($T_{100\%}$)	E (rea.)	E (rea.)	E (veln)	E (vor)
1	Cr 51 EDTA	0.293	0.249	0.233		
2	Cr 51 EDTA	0.203	0.156	0.148		
3	Cr 51 EDTA	0.281	0.304	0.298		
4	Cr 51 EDTA	0.430	0.373	0.350	0.398	0.37
5	Cr 51 EDTA	0.297	0.290	0.283	0.273	0.27
6	Cr 51 EDTA	0.432	0.448	0.438	0.450	0.43
7	I 131-thalamat	0.287	0.273	0.269	0.306	0.30

CDC calculated from plasma flows obtained by curve resolution

1	Cr 51 EDTA	4.91	4.06		
2	Cr 51 EDTA	3.69	3.53		
3	Cr 51 EDTA	3.69	3.69		
4	Cr 51 EDTA	4.07	3.22	4.38	3.62
5	Cr 51 EDTA	5.13	4.37	4.63	3.94
6	Cr 51 EDTA	6.44	5.95	5.27	4.90
7	I 131-thalamat	4.94	4.17	7.44	6.32
n		6	6	3	3
x		4.96	4.47	4.76	4.05
\pm SE		± 0.39	± 0.46	± 0.26	± 0.18

CDC calculated from directly measured plasma flows

1	Cr 51 EDTA	4.88	4.03	3.31	
2	Cr 51 EDTA	4.71	3.52	3.38	
3	Cr 51 EDTA	3.53	3.90	3.87	
4	Cr 51 EDTA	5.95	4.94	3.91	5.37
5	Cr 51 EDTA	4.30	4.18	3.46	3.89
6	Cr 51 EDTA	5.84	6.13	5.85	6.17
7	I 131-thalamat	5.54	5.22	4.41	5.98
n		6	6	6	3
x		4.87	4.43	3.96	5.14
\pm SE		± 0.47	± 0.39	± 0.39	± 0.67

3) The bolus input expts. performed with the venous catheter alone showed similar bended curve shapes, and the transit time for the whole curve was equal to the venous blood flow ratio. Convolution of the intravascular curve part obtained from residue curve with the catheter response curves obtained at equal blood flow values yielded curve shapes equal to the venous outflow curves for intravascular indicators. Deconvolution of $T_{100\%}$ -albumin curves with the catheter response curves gave curve shapes almost identical to the intravascular curve part of the residue curves. Comparison of $T_{100\%}$ -albumin curves with the intravascular curve part of venous curves showed almost identical shapes of these curves. These comparisons of curve shapes were used as indirect tests of the reliability of the reconvolution procedures employed in the main series of expts.

4) The vascular volume of distribution was determined in 6 expts. with Cr 51 labeled erythrocytes and in 2 expts. with $T_{100\%}$ -albumin as indicator. The intravascular volume

TABLE III Volumes of distribution for Cr-51-EDTA and I-131-thalamate calculated from residue and out flow curves. Average \pm SE is given below for 7 and 4 expts.

Total volume of distribution (ml/100 g)		Intravascular volume of distribution (ml/100 g)		Extravascular volume of distribution (ml/100 g)		"Fical" volume of distribution ¹⁰⁰ (ml/100 g)	
Res	Voin	Res	Voin	Res	Voin	Res	Voin
10.6		1.6		9.0		2.9	
9.0		1.9		7.1		2.4	
15.9		1.5		14.4		3.8	
11.3	11.9	2.3	2.3	9.0	9.6	4.8	5.3
16.4	17.1	1.5	1.6	14.9	15.5	5.9	6.8
19.7	20.2	1.9	2.3	17.8	17.9	6.3	7.3
16.7	19.4	2.2	1.6	14.5	17.8	9.1	9.7
7	4	7	4	7	4	7	4
14.2	17.2	1.8	2.0	12.4	15.2	5.4	7.0
IE ± 1.3	± 1.9	± 0.1	± 0.2	± 1.5	± 1.9	± 0.9	± 1.0

¹⁰⁰ the extravascular plasma volume.

the volume of distribution defined by the final outflow rate.

measured by the two indicators gave for all 8 expts. expressed in per cent of muscle weight average value \pm SE. 3.0 ± 0.4 ml/100 g. As mentioned previously this value was used in the calculation of plasma flow from the intravascular curve parts.

Discussion

Methodological considerations

The fulfillment of the assumptions mentioned in theoretical considerations is considered in the following:

1. The indicator Cr-51-EDTA is inert. Thus conservation of matter is fulfilled for this indicator. This is presumably also valid for I-131-thalamate.
2. Stationarity of blood flow was evaluated from the directly measured blood flow. Only minor differences were observed between the initial and the average values, indicating a minimum perturbation effect of the injection. The standard error of the average plasma flows was from about 2 to 10 per cent, which is considered an acceptable approximation to stationarity of flow during the expts.
3. Linearity of the system is probable as the indicators used were inert and on this basis the response of the system should be proportional to the input. No observations seem to indicate non-linearity of the system.
4. Equivalent entry is presumably obtained by the fast injection of the bolus in a direction transversal to the blood stream.
5. Inefficient counting of the indicator particles in the different volumes of distribution must be obtained as the tissue elements (cells, interstitial space and capillaries) are equally mixed in all tissue regions. However there is as mentioned no demand of efficient counting from one region to another in the tissue.

6 Recirculation was completely eliminated by the experimental technique.

7 The measurement of the height of the registered curve was examined from the experiments where the duration of the input and the shortest transit time of the system were measured at different blood flow values. The analysis indicates that a time interval of 2 to 5 exists in which measurement of the correct height of the curve is possible at the blood flow values actually used.

8 Considering the relatively great diffusion velocity for the indicator in water and the relatively short diffusion distances in a considerable part of the interstitial space close to the capillaries, it is reasonable to expect that the indicator is relatively well mixed in this "subcompartment". Consequently it can be expected that the initial phase of the washout of the extracted fraction will be dominated by this washout process in the first minutes yielding a possibility for a relatively exact extrapolation by a monoexponential function.

The extrapolation of the extravascular curve part back to peak time was evaluated experimentally by comparison of plasma flow values measured directly in the initial phase and plasma flow values calculated from the intravascular volume of distribution and the mean transit time of the intravascular curve part obtained by extrapolation and subtraction. These results show no significant difference. These results are not incompatible with the statement that the extrapolation procedure employed has been correct. Also a comparison of the curve configurations was performed. The curves were dominated by a monoexponential washout function over more than 95 per cent and they showed good agreement which is taken as a substantial support for the reliability of the extrapolation procedure employed. It is important to note that all indicator molecules which pass the interstitial space during their transit of the muscle will be considerably delayed compared to molecules remaining in the vascular space during the transit. In average this delay was a decrease in transport velocity of a factor 26 for molecules permeating the capillary membrane, and even the fastest extravascular molecules had a washout rate which was at least a factor 7 slower than the slowest intravascular washout rate. This is good conditions for a separation of the two fractions by curve resolution.

The results show that the CDC values are rather insensitive of the extrapolation procedure used as both the extraction and the intravascular mean transit time are changed in the same way by different extrapolation procedures which reduces the combined effect to a minimum. On this basis the CDC values calculated from plasma flow obtained by curve resolution presumably are to be preferred for those calculated from the directly measured flow.

9 The total area under the curves was obtained by monoexponential extrapolation to infinity. The procedure was examined in a previous article (Lassen and Sejrsen 1971). The final slopes were not significantly different by the two measurements within a single experiment, indicating that the final slowest washout rate was obtained. This yields the possibility to extrapolate correctly to infinity (Lassen and Sejrsen 1971).

10 The different "Taylor diffusion effect" for test and reference molecules was taken into account by the area calculation which tends to reduce this difference (Lassen and Crone 1970). This assumption is only of importance for the indicator diffusion method, as the indicator serves as its own intravascular reference for the methods based on kinetic analysis.

1. Back diffusion of indicator from the extravascular space to the blood must be negligible in the initial phase to give the correct extraction value by the indicator diffusion method. Support for the fulfillment of this assumption has been taken from the existence of a horizontal part in the extraction curve (see Fig. 4). This horizontal curve part has been interpreted as the initial time interval, where back diffusion from tissue to blood presumably is negligible (Lassen and Crone 1970). Back diffusion is of no importance in the four kinetic methods as the retropolation procedures gives a description of this process and by this minimize the effect at peak time.

From these considerations the assumptions of the methods seem to be reasonably fulfilled in the present experiments.

The extravascular curve part showed a bended shape which in average for all 7 experiments a factor of 12.8 between the rate constants of the early and the final part of this curve. The connection inhomogeneity of perfusion has to be considered. In previous studies with washout of Xenon-133 from local sites of the tissue, no signs of gross inhomogeneity of blood flow were found in the same muscle preparation at high and low blood flow values (Sørensen and Tønnesen 1968 a, Tønnesen and Sejrsen 1970). Also by an intravascular indicator Iodide-131-albumin, no signs of a non uniform distribution of blood flow were obtained for the same muscle preparation (Sejrsen and Tønnesen 1968 b). In the blood perfused sternomastoid muscle in dogs a rather homogeneous uptake of D_2O was demonstrated (Jørgensen *et al.* 1959) which also indicates a rather homogeneous blood perfusion. In the present study the intravascular curves were dominated by a monoexponential part constituting about 96.1 ± 1.7 per cent (average \pm SE, $n = 7$) of the washout as obtained from T_{1/2}-albumin curves. The rest can presumably be explained by the presence of small amounts of connective tissue in the preparation and the parabolic shape of the flow profile in greater vessels within the muscle preparation and the effect of the catheter of the results obtained in the experiments with perfusion of the catheter. Thus a rather high degree of homogeneity of blood flow in the capillaries and per gram of tissue mass seems to be evident from the present observations and the grossly bended shape of the extravascular curve part cannot be explained by inhomogeneity of blood flow. The consequence of this is that the main assumptions for the osmotic transient method (Pappenheimer *et al.* 1951) which is a single well mixed extravascular space obviously is not fulfilled as the extravascular curve part is not monoexponential throughout its course.

Agreement was found between the permeability data obtained by the indicator diffusion method and the four methods based on kinetic analysis of the curves recorded by residue and outflow detections after bolus injection. This can be interpreted as a mutual support of the methods. The Taylor diffusion effect tends to give an overestimation of the extraction fraction as measured by the indicator diffusion method. The extrapolation procedures performed when using the bolus injection, kinetic analysis methods were considered to affect an overestimation of the extraction fraction. Theoretically a correction of the extracted fraction is needed as previously proposed (Sejrsen 1969 and Rakic *et al.* 1976) for partial in series models. But the correction constitute only a decrease of the E value of about 3 to 4 per cent and of the CDC value of about 1 per cent which is considered insignificant, and no correction has been performed. The distinctive difference between the

results obtained by the two types of methods indicates small or insignificant importance of the "Taylor diffusion effect"

The agreement of extraction and CDC values found for the transport process in both directions over the capillary membrane supports the concept that the capillary membrane functions as a symmetrical membrane for transport of small hydrophilic indicators. This finding is supported by the results of washout of Cr 51 EDTA after local injection in muscle in man compared to results obtained by the indicator diffusion method (Trap-Jensen, Korsgaard and Lassen 1970, Trap-Jensen and Lassen 1971).

Capillary permeability coefficients

Calculation of the permeability coefficient P_a (cm/s) for Cr 51 EDTA from the extraction and CDC values obtained by the indicator diffusion method and an overall average of the results from the other methods using calculated or directly measured plasma flows, and a capillary surface area of 7 000 cm²/100 g of tissue (Pappenheimer *et al.* 1951) gives P_a values of $1.16 \cdot 10^{-4}$ cm/s (n = 6), $1.10 \cdot 10^{-4} \pm 0.05 \cdot 10^{-4}$ cm/s (n = 18) and $1.05 \cdot 10^{-4} \pm 0.06 \cdot 10^{-4}$ cm/s (n = 18), respectively. The permeability calculated from the tracer kinetic methods for passage from blood to tissue gives $1.16 \cdot 10^{-4} \pm 0.07 \cdot 10^{-4}$ cm/s (n = 9) and from tissue to blood $1.03 \cdot 10^{-4} \pm 0.08 \cdot 10^{-4}$ cm/s (n = 9). These permeability values are in agreement with data obtained with the same preparation and method by Paaske (1977 a), who found a value of $1.02 \cdot 10^{-4}$ cm/s at a plasma flow of about 15 ml/100 g min.

Crone (1963) used the indicator diffusion method for determination of the permeability of sucrose in dog hind limbs which were considered predominantly to consist of muscle tissue. Autoperfusion was performed at blood flows estimated to about 7 ml/100 g min but this parameter was not measured. A P_a value of $0.74 \cdot 10^{-4}$ cm/s was obtained for sucrose which has almost the same molecular weight as Cr 51 EDTA. However, the calculation of the CDC value was performed from equation (14) using blood flow instead of plasma flow. The concentration gradient over the capillary membrane in the initial phase was taken to be equal to the logarithmic mean concentration in the capillary. To represent the driving force for transcapillary diffusion the concentration has to be that in plasma water and consequently plasma water flow should be used for calculation. Correction for this gives a value of about $0.37 \cdot 10^{-4}$ cm/s. This figure is about one half of those for muscle tissue obtained proper at the plasma water flow level used (Paaske 1977 a). The discrepancy can be explained by the fact that the hind limb contains various tissues and furthermore shunt flow in the paw.

Rippe *et al.* 1978 found by the indicator diffusion method on the rat hindquarter preparation during artificial perfusion and maximum vasodilatation a CDC value for Cr 51 EDTA of 5.67 ml/100 g min which corresponds to a P_a value of $1.35 \cdot 10^{-4}$ cm/s. This value is little lower than the results obtained for the autoperfused cat gastrocnemius muscle during maximum exercise which was about $2.1 \cdot 10^{-4}$ cm/s (Paaske 1977 a).

Another investigation of permeability of Cr 51 EDTA has been performed by the indicator diffusion method in the exercising human forearm with an inflated cuff around the forearm to avoid influence from tissues in the hand (Trap-Jensen and Lassen 1970, Trap-Jensen 1970, and Trap-Jensen and Lassen 1971). Blood samples were taken from a profound

the control form. Also in this investigation the region under study was taken to consist of a unit of muscle tissue. P values of $0.77 \cdot 10^{-6}$ to $0.88 \cdot 10^{-6}$ cm/s were found at plasma flows of 14 to 18.4 ml/100 g min. These results are closer to those obtained for the perfused rat gastrocnemius muscle at equal flow range. In this connection it is important to remember that also in the mentioned two studies performed on rat hindquarter and human arm the relative amount of other tissue under study which is not muscle tissue, is considerably larger than in the gastrocnemius muscle preparation. Excluding skeletal muscle tissue has a higher capillary permeability than the other tissues included as shown by Paaske (1977 a, 1977 b, and 1977 c), Paaske and Nielsen (1976).

In all calculations of P values a capillary surface area of 7 000 cm²/100 g of tissue has been employed. As shown by Paaske (1977) recruitment of capillaries takes place with a factor of 4 at a plasma flow change from 2 to 50 ml/100 g min. The number of capillaries in a section per cm² is about 100 during rest and 400 during maximum exercise (Landis and Speitelmeier 1963, Hammersten 1968, Eriksson and Myrberg 1972, Brodal *et al* 1977). Calculation of the capillary surface area from these figures and an average value of the capillary diameter of 6.5 μ m and the length of 1 200 μ m gives a capillary surface area of about 2 500 and 10 000 cm²/100 g of tissue, respectively. At a plasma flow of 15 ml/100 g min an area of 5 000 cm²/100 g presumably is a better estimate to use, resulting in a correction of the P_c values to figures around $1.5 \cdot 10^{-6}$ cm/s.

The free diffusion coefficient in water D for Cr 51 EDTA is about $0.7 \cdot 10^{-5}$ cm²/s. From the formula $P = D/\Delta x$, where Δx is "the membrane thickness" it is possible to express Δx as the total path length across the membrane which becomes about 0.5 cm in 100 g of muscle. As the capillary membrane is about 1 000 Å in thickness this means that the effective area constitute about 1/50 000 of the capillary surface area.

Methodological possibilities

bolus injection, residue or venous registration method for measurement of capillary permeability using only a test substance (Sejrsen 1970) has been employed on other tissues such as brain (Eichling *et al* 1974 and Raichle *et al* 1976), cutaneous, subcutaneous, and skeletal muscle tissue (Paaske 1976, 1977 a, b, c, and Paaske and Nielsen 1976) by residue section and as a study of C 14-inulin permeability in skeletal muscle (Paaske and Sejrsen 1977) by venous detection. It is presumably possible to use even larger indicator molecules than albumin which makes the method suitable for the study of the equivalent pore size of the capillary membrane for transport of hydrophilic molecules, and the extracellular volume of distribution for albumin. If the injected dose is chosen to be sufficiently high, it is possible to measure and extrapolate the extravascular curve to peak time even at low extraction values. The extracted fraction of the indicator will have a very slow wash-out rate in the direction from tissue to blood. This gives the possibility to determine the activity level of the extracted fraction from a slowly falling curve which can be registered over a long time interval, which compensates for the low counting value.

In contrast the indicator diffusion method has its limitation for indicators as large as albumin in skeletal muscle at a plasma flow level of 5 to 10 ml/100 g min, for which the relative concentration difference between test and reference indicator becomes indistinct due to

the inaccuracy of both concentration measurements *i.e.* at low values of E . Thus it is impossible to measure albumin extraction by this method even if labelled erythrocytes could be used as reference indicator.

The bolus injection residue detection method is due to the simplicity of the method suitable for clinical purposes. Recirculation and background activity can often be measured on a symmetrical region and subtracted from the registered curve.

Volumes of distribution

The volumes of distribution determined by kinetic analysis for the hydrophilic indicators used are in accordance with results obtained in previous studies (Lassen and Sejrnsen 1977; Paaske and Sejrnsen 1977). Difference in volume of distribution between sucrose and inulin was found by Crone and Garlick (1970), but edema formation and insufficient time for equilibration in these experiments can presumably explain these results. Krukhoffer (1946a, b) found also almost identical volumes of distribution for sucrose and inulin in nephrectomized rabbits.

The monoexponential final part of the washout curves constitute a considerable fraction, almost 50 per cent, of the extravascular volume of distribution. For C 14-inulin the corresponding value was found to be about 76 per cent (Paaske and Sejrnsen 1977). In muscle tissue about one half of the interstitial space is located inside the longitudinal tubules of the sarcoplasmic reticulum (Porter and Palade 1957), which might be considered to account for the well mixed compartment responsible for the final monoexponential washout. The sarcoplasmic reticulum consists of transversal and longitudinal tubules. Only longitudinal tubules have a limiting membrane to the other part of the interstitial space. The great distance within the longitudinal tubules is about 2 μm and complete mixing by diffusion is obtained for the actual indicators over this short distance within a few ms. Thus the final monoexponential curve part could indicate the washout from the sarcoplasmic reticulum and then these final rate constants presumably is determined by the permeability of the membrane of the lateral saccus in the longitudinal tubules. The total area of this membrane is from histometrical observations estimated to be about 6 times greater than the capillary membrane at a plasma flow of 15 ml/100 g min (Porter and Palade 1957). Calculation of the permeability from $E(\text{tail}) = \bar{E}(\text{total})/\bar{E}(\text{tail})$ leads to a sarcoplasmic diffusion capacity $\text{SDC} = -f_p / 0.89 \ln(1 - E) = 0.48 \text{ ml/100 g min}$. This value is about 10 times lower than that obtained for the capillary membrane at a plasma flow of 15 ml/100 g min. The permeability for the sarcoplasmic reticulum $P_d(\text{SR})$ then becomes about $2.6 \cdot 10^{-6} \text{ cm/s}$ which is about 60 times lower than the permeability of the capillary membrane at that flow rate and with a range from about 30 at rest to 120 at maximum exercise.

Autoradiography is suggested as a suitable method for examination of the validity of the hypothesis for the final washout rate constant. Supported by such examinations this kinetic analysis will constitute another attempt to gain information about a subsystem inside the main system by use of kinetic analysis.

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References

- DAL, P. F. IVORIE and L. HARRAMON, Capillary supply of skeletal muscle fibers in untrained and endurance-trained men. *Amer J Physiol.* 1977 232 (6), H705-H712.
- NARD, H. P. G. J. YOUNG and T. ENGE, Transcapillary exchange of water and of other substances: certain organs of the dog. *Amer J Physiol.* 1955, 183 221-234.
- RE, C. The permeability of capillaries in various organs as determined by use of the "indicator diffusion" method. *Acta physiol. scand.* 1963, 58 292-305.
- RE, C. Capillary Permeability—Techniques and problems. In *Capillary Permeability* (Alfred Benzon Symp. 10), C. Crone and N. A. Lassen (Eds.) Munksgaard, Copenhagen, 1970, pp. 15-31.
- RE, C. and E. GALLICK, The penetration of insulin, sucrose, mannitol and trinitrophenol from the interstitial space in muscle into the vascular system. *J Physiol. (Lond.)* 1970 210, 387-404.
- SLING, J. D. M. E. RAICHLE, R. L. GROSS, JR. and M. M. TEN-PPOONIAN, Evidence of the limitations of a freely diffusible tracer in brain of the Rhesus monkey. *Cerebral Res.* 1974, 35, 358-364.
- STROM, E. and B. MYRBERG, Microvascular dimensions and blood flow in skeletal muscle. *Acta physiol. scand.* 1972, 86, 211-222.
- WIKSTRÖM, F. The pattern of the terminal vascular bed and the ultrastructure of capillaries in skeletal muscle. In *Oxygen Transport in Blood and Tissue* (Stuttgart, Thieme, 1968, pp. 184-197).
- WIKSTRÖM, F. Insulin as an indicator for the extracellular space. *Acta physiol. scand.* 1946 a, 11 16-36.
- WIKSTRÖM, F. The significance of diffusion and convection for the distribution of solutes in the interstitial space. *Acta physiol. scand.* 1946 b, 11 57-67.
- WIKSTRÖM, F. and J. R. PAPPENHAGEN, Exchange of substances through the capillary walls. In *Handbook of Physiology: Circulation*, Washington, D.C., Am. Physiol. Soc., 1963, sect. 2, vol. II, chap. 29 pp. 61-103A.
- WIKSTRÖM, N. A. and C. CRONE, The extraction fraction of capillary bed as hydrophilic molecules: Theoretical considerations regarding the single injection technique with discussion of the role of diffusion between plasma streams (Taylor's effect). In: *Capillary Permeability* (Alfred Benzon Symp. 11), C. Crone and N. A. Lassen (Eds.) Munksgaard, Copenhagen, 1970, pp. 44-59.
- WIKSTRÖM, N. A. and P. SÖDERSTRÖM, Monophasic extrapolation of tracer clearance curves as kinetic analysis. *Cerebral Res.* 1971, 29 76-87.
- WIKSTRÖM, N. A. and J. TRAN-JENSEN, The validity of the indicator diffusion method for measuring the capillary diffusion capacity for ^{51}Cr EDTA in hyperanaemic skeletal muscle. *Europ J clin. Invest.* 1970, 1 118-123.
- WIKSTRÖM, W. F. Capillary permeability in cutaneous tissue. *Acta physiol. scand.* 1976, 98 492-499.
- WIKSTRÖM, W. F. Capillary permeability in skeletal muscle. *Acta physiol. scand.* 1977 a, 101 1-14.
- WIKSTRÖM, W. F. Absence of restricted diffusion in cutaneous capillaries. *Acta physiol. scand.* 1977 b, 100 333-339.
- WIKSTRÖM, W. F. Absence of restricted diffusion in adipose tissue capillaries. *Acta physiol. scand.* 1977 c, 100 430-436.
- WIKSTRÖM, W. F. and S. L. NIELSEN, Capillary permeability in adipose tissue. *Acta physiol. scand.* 1976, 98 116-122.
- WIKSTRÖM, W. F. and P. SÖDERSTRÖM, Transcapillary exchange of ^{14}C -urea by free diffusion in channels of fixed vessels. *Acta physiol. scand.* 1977 100, 437-445.
- WIKSTRÖM, J. R. E. M. REICH and L. M. BORRERO, Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Amer J Physiol.* 1951 167 13-46.
- WIKSTRÖM, K. R. and G. E. PALADE, Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells. *J biophys. biochem. Cytol.* 1957 3 269-299, incl. Plates 83-94.
- WIKSTRÖM, M. P., J. O. EICHLING, M. G. STRAATHMAN, M. J. WELCH, K. B. LARSEN and M. M. TEN-PPOONIAN, Blood-brain barrier permeability of ^{14}C -labeled alcohols and ^{18}O -labeled water. *Amer J Physiol.* 1976, 230 543-552.
- WIKSTRÖM, B. A. KAWY and B. POLKOW, Simultaneous measurements of capillary diffusion and filtration exchange at alterations in the capillary permeability-surface area product (PS) and during shifts in filtration-absorption. *Acta physiol. scand.* 1978, 102, 32A-33A.
- WIKSTRÖM, P. Blood flow in cutaneous tissue in man studied by infusion of radioactive Xaeson. *Cerebral Res.* 1969 21 215-229.

- SEJRSSEN, P. Single injection external registration method for measurement of capillary function. *Capillary Permeability* (Alfred Benzon Symp. II), C. Crone and N. A. Lassen (Eds.) Munksgaard, Copenhagen 1970 pp. 256-60.
- SEJRSSEN, P. and K. H. TONNESSEN, Inert gas diffusion method for measurement of blood flow using a perfusion technique. Comparison with directly measured blood flow in isolated gastrocnemius muscle of cat. *Circulat Res* 1968 **22**: 679-693.
- SEJRSSEN, P. and K. H. TONNESSEN, Uniform distribution of blood flow in skeletal muscle. *Acta physiol scand* 1968 **5**: 73-83A.
- THOMPSON, A. M., H. M. CAVERT, N. LUSON and R. L. EVANS, Regional tissue uptake of D_2O in perfused organs: rat liver, dog heart and gastrocnemius. *Amer J Physiol* 1959 **197**: 897-90.
- TONNESSEN, K. H. and P. SEJRSSEN, Washout of ^{133}Xe after intramuscular injection and determination of blood flow in skeletal muscle. *Scand J clin. Lab Invest* 1970 **25**: 71-81.
- TRAP-JENSEN, J. Increased capillary permeability to ^{125}I iodide and ^{51}Cr EDTA in the exercising legs of long-term diabetics. *Clin. Sci* 1970 **39**: 39-49.
- TRAP-JENSEN, J. and N. A. LASSEN, Capillary permeability for smaller hydrophilic tracers in normal skeletal muscle in normal man and in patients with long-term diabetes mellitus. In *Capillary Permeability* (Alfred Benzon Symp. II), C. Crone and N. A. Lassen (Eds.), Munksgaard, Copenhagen, 1970, 135-152.
- TRAP-JENSEN, J. and N. A. LASSEN, Restricted diffusion in skeletal muscle capillaries in man. *Am J Physiol* 1971 **220**: 371-376.
- TRAP-JENSEN, J., O. KORSGAARD and N. A. LASSEN, Capillary permeability in human skeletal muscle measured by local injection of ^{51}Cr EDTA and ^{133}Xe . *Scand J clin. Lab Invest* 1970 **25**: 93-99.
- ZIERLER, K. L., Equations for measuring blood flow by external monitoring of radiolabeled tracers. *Circ Res* 1965 **16**: 309-311.

Gastric acid secretion and water balance in the marine teleost *Gadus morhua*

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HOLSTEIN, B. Gastric acid secretion and water balance in the marine teleost *Gadus morhua*.

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Gastric acid secretion was monitored in unanesthetized codfish surgically equipped with catheter draining stomach. The pylorus was ligated. Without perfusion of the intestine, or during perfusion with pure water (100 % SW) or slightly diluted (87 %) SW gastric acid secretion was low ($<10 \mu\text{mol H}^+/\text{kg h}$) the fishes became dehydrated during the experiment. Perfusion of the intestine with 90% or 33% SW led the fishes to compensate for the water loss to the environment and greatly enhanced gastric acid release. Acid secretion was elevated also in fishes in which the dehydration was prevented by an intrascular infusion of saline. Perfusion of the intestine (0.5 ml/h) decreased the hourly efficient volume (ionic rise) from the stomach to almost zero within 2 h. The effect was independent of the degree of tone of the perfusing SW but was less pronounced when the perfusion rate was lowered. Intrascular saline also depressed drinking rate. The acid secretion prevailing during perfusion of the intestine with an isotonic preventing dehydration—suggested to represent the basal acid secretion of the codfish—was inhibited by atropine, hexamethonium, and pentobarbital.

marine fish loses water to the hyperosmotic environment, and to compensate for this, has to drink continuously (Smith 1930). This drinking was utilized in a technique developed in this laboratory (Holstein 1975) for *in vivo* studies of the regulation of gastric acid secretion in the Codfish. Under MS-222 anesthesia polyethylene cannula was placed in the mouth so that the water swallowed by the fish, mixed with the gastric secretions, could be collected. The method was subsequently modified to allow for correction of losses through the oesophagus (Holstein 1977). The surgical procedure included the ligation of pylorus.

As the uptake of water takes place in the intestine, it was suspected that the pylorus-ligated fishes might suffer from dehydration. This has been confirmed by weighing fishes at the end of the experiments, i.e. on the third day post-surgically the BW was then $87.9 \pm 5\%$ (mean and S.E. of 40 animals) of the presurgical value. This report describes a technique for the *in vivo* perfusion of the intestine. It was found that the fishes could compensate for the water loss provided that the sea-water used for the perfusion was sufficiently diluted, and that this was accompanied by a profoundly increased secretion of acid from the stomach. Some observations on drinking rate are also reported.

Materials and Methods

Codfishes of both sexes, weighing between 290 and 730 g were used. Before use, the fishes were kept on 1 food for at least one week in the aquarium (10°C). Surgery was carried out under MS-222 (or anesthesia as described earlier (Holstein 1975). The following catheters were positioned (cf. Fig. 1):

1. *Intestinal perfusion catheter (C1)*. The catheter was made from polyethylene tubing (PE 50, I. medic), its back end flared to prevent it from sliding through the intestinal wall. To aid the positioning, the leading end of the catheter was equipped with a 0.8 mm needle. Through an incision made a few cm proximal to the pyloric appendices, the needle was inserted into the gastro-intestinal lumen, passed the appendices and taken through the intestinal wall (Fig. 1 A). The accompanying PE 50 was then, until the flange was stopped by the luminal surface. The catheter, previously permanently bent into C heating a few mm from the flange, was secured by a purse-string suture to the muscular coat of the wall.

2. *Catheter draining the stomach (C2)*. The catheter was made by flaring a piece of PE 200 tubing. A perspex sond was used to introduce the catheter into the stomach by way of the mouth. The procedure has been described in detail (Holstein 1975), but was slightly modified. Instead of taking the catheter through the intestinal wall, it was now exteriorized through the incision proximal to the pyloric appendices (Fig. 1 A, B). The ligatures L1 and L2 were then tied on either side of the incision.

A. alternative technique to drain the stomach was suggested by Cederberg of this Institute. A cannula was made from PE 200 tubing by flaring and imposing a permanent bend 5 mm from the tip (C'2, Fig. 1 C). The cannula was positioned by a technique analogous to the one used for C3 (but with a coarser needle (1.4 mm) and a piece of PE 200 aided the positioning. A tygon tubing was used to connect the cannula to a fraction collector.

3. *Catheter for perfusion of the stomach (C3)*. Through the mouth a straight perspex sond was inserted into the stomach (Fig. 1 C). A piece of PE 50 tubing was attached to the blunt end of an injection needle and the needle inserted through the stomach wall, into the lumen and into the sond. The tubing was positioned until the needle was viable outside the mouth, the sond removed and C3—constructed as C—attached to the needle. By retracting the PE 50 tubing the flange of C3 was positioned against the inner surface of the anterior part of the stomach. A purse-string suture secured the catheter in the muscular coat of the stomach. The catheters C1, C2 or C'2, and C3 were exteriorized through stab wounds in the body wall, and the body wall closed by continuous suture. C2/C'2 was connected to a fraction collector for the outflow, one hour fractions, and C1 and C3 to roller pumps.

Experimental schedule. The surgery was carried out during the a.m. and following recovery from anesthesia the fishes were placed in separate tanks and the catheters connected as described. Perfusion of intestine when employed, started at once or in some initial studies on the morning of the second day. Unless otherwise stated the perfusion rate was 8.5 ml/h (corresponding to 1/10 of the body weight of the fish). Perfusion fluid was either sea water (100 SW) or SW diluted with distilled water to 67%, 50% or 33% SW. Perfusion of the stomach started 20–22 h following surgery. The perfusion fluid was 100% containing 100 mg/l phenol red (PR), and was delivered at 8.5 ml/h.

In the experiment with secretory antagonists, these were administered during the second day. A Perfusor IV infusion Pump was employed for the intraluminal perfusion of the drugs (0.62 ml/h).

When interrupting the experiments, a blood sample (3–6 ml) was drawn from the caudal vein in a syringe moistened with heparine. Eve fluid was taken from the intestine and the fish weighed.

Analyses on gastric effluence. 2-ml aliquots of the effluent fluid from the stomach was titrated (4-tiltrotor assembly Radiometer Copenhagen) with 0.05 M sodium hydroxide. End point of the titration was the pH of the aquarium water (samples obtained before perfusion of the stomach with PR) or the pH of the solution used for perfusion of the stomach (during perfusion with PR). The acid content of the fraction was calculated from acidity and sample volume. The concentration of PR in each fraction was determined by reading the optical density (463 nm, isobestic point, cf. Öbrink 1948) of 200 µl of the fraction diluted with 2.0 ml 0.1 M HCl. A identically diluted aliquot of the perfusion fluid served as blank. The recovery of PR was then calculated and considered to mirror the recovery of secreted acid. In experiments where PR was used only secretory data corrected for this recovery are presented. In experiments without PR, uncorrected values of acid output are given. Acid output is expressed as µmol/kg h. The BW obtained before surgery was used to calculate this.

For each animal, also the mean hourly recoveries of PR and the volume of the gastric perfusate for whole perfusion period were calculated.

Analyses on blood plasma. Following centrifugation of the blood the plasma samples were stored for

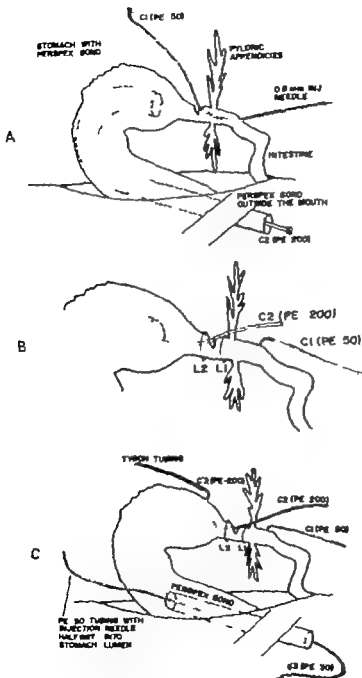


Fig. 1. A-C. Schematic illustration of the surgical procedure. Parts of catheters or food drawn in by dashed lines are above the gastro-intestinal lumen.

Materials and Methods

Codfishes of both sexes, weighing between 290 and 730 g were used. Before use the fishes were kept out of food for at least one week in the aquarium (10°C). Surgery was carried out under MS-222 (or anesthesia as described earlier (Holstein 1975)). The following catheters were positioned (cf. Fig. 1):

1. *Intestinal perfusion catheter (C1)*. The catheter was made from polyethylene tubing (PE 90, Medic), its back end flared to prevent it from sliding through the intestinal wall. To aid the positioning, the leading end of the catheter was equipped with a 0.8 mm needle. Through an incision made in the proximal to the pyloric appendices, the needle was inserted into the gastro-intestinal lumen, past the appendices and taken through the intestinal wall (Fig. 1 A). The accompanying PE 90 was then pushed until the flange was stopped by the luminal surface. The catheter previously permanently bent (60°) heating a few mm from the flange, was secured by a purse-string suture in the muscular coat of the stomach.

2. *Catheter draining the stomach (C2)*. The catheter was made by flaring a piece of PE 200 tubing. A perspex pond was used to introduce the catheter into the stomach by way of the mouth. The procedure has been described in detail (Holstein 1975), but was slightly modified. Instead of taking the catheter through the intestinal wall, it was now exteriorized through the incision proximal to the pyloric appendices (Fig. 1 A-B). The larynx L1 and L2 were then tied on either side of the incision.

An alternative technique to drain the stomach was suggested by Cederberg of this institute. A cannula was made from PE 200 tubing by flaring and imposing a permanent bend 5 mm from the tip (C2', Fig. 1 C). The cannula was positioned by a technique analogous to the one used for C2 (but with a coarser needle (1.4 mm) and a piece of PE 200 aided the positioning. A tygon tubing was used to connect the cannula to a fraction collector.

3. *Catheter for perfusion of the stomach (C3)*. Through the mouth a straight perspex pond was inserted into the stomach (Fig. 1 C). A piece of PE 90 tubing was attached to the blunt end of an injector and the needle inserted through the stomach wall, into the lumen and into the pond. The tubing was pushed until the needle was visible outside the mouth, the pond removed and C3—constructed as C2—attached to the needle. By retracting the PE 90 tubing the flange of C3 was positioned against the luminal surface of the anterior part of the stomach. A purse-string suture secured the catheter in the muscular coat of the stomach. The catheters C1, C2 or C2', and C3 were exteriorized through stab wounds in the body wall, and the body wall closed by continuous suture. C2/C2' was connected to a fraction collector for the outflow in one hour fractions, and C1 and C3 to roller pumps.

Experimental schedule. The surgery was carried out during the a.m. and following recovery from anesthesia the fishes were placed in separate tanks and the catheters connected as described. Perfusion of the intestine when employed, started at once or at some initial studies on the morning of the second day. Unless otherwise stated, the perfusion rate was 8.5 ml/h (corresponding to 'high rate' of *Neosalan*). Perfusion of the stomach started 20–22 h following surgery. The perfusion fluid was 100 ml containing 100 mg/l phenol red (PR), and was delivered at 8.5 ml/h.

In the experiments with secretory antagonists, these were administered during the second day. A Perfusion IV Infusion Pump was employed for the i.m. injection of the drugs (0.62 ml/h).

When interrupting the experiments, blood samples (3–6 ml) were drawn from the caudal vein with a syringe moistened with heparine. Eventual water in the intestine was drained and the fish weighed.

Analyses on gastric effluence. 2-ml aliquots of the effluent fluid from the stomach was titrated with a titration assembly Radiometer (Copenhagen) with 0.05 M sodium hydroxide. End point of the titration was the pH of the aquarium water (samples obtained before perfusion of the stomach with PR) or the fraction was calculated from acidity and sample volume. The concentration of PR in each fraction was determined by reading the optical density (463 nm, isosbestic point, cf. Öbrink 1948) of 200 µl aliquot diluted with 2.0 ml 0.1 M HCl. An identically diluted aliquot of the perfusate fluid served as a standard. The recovery of PR was then calculated and considered to mirror the recovery of secreted acid. In experiments where PR was used, only secretory data corrected for this recovery are presented. In experiments without PR, uncorrected values for acid output are given. Acid output is expressed as $\mu\text{mol kg}^{-1} \text{ h}^{-1}$ the BW obtained before surgery was used to calculate this.

For each animal, both the mean hourly recoveries of PR and of volume of the gastric perfusate for the whole perfusion period were calculated.

Analyses on blood plasma. Following centrifugation of the blood, the plasma samples were stored for

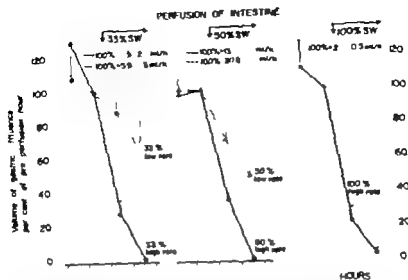


Fig. 3. Effect of intestinal perfusion on volume of gastric effluence. Different rates of intestinal perfusion used, but calculated per kg of total BW (these were, in ml/kg \pm S.E. (33% high rate), 3.7 ± 0.6 (low rate), 18.6 ± 2.3 (50% high rate), 2.9 ± 0.3 (50% low rate), and 13.6 ± 0.4 (100% high rate)).

The perfusion of the intestine affected the effluent volume from the stomach. In experiments in which the intestinal perfusion started immediately following recovery from anaesthesia (and the perfusion of PR about 20 h later) most of the 20 first fractions were empty or occasionally a few drops were collected (not illustrated). In some other series the perfusion of the intestine commenced at about 20 h postsurgically and PR 2 h later. From Fig. 3 it will be seen that there was a drastic decrease in effluent volume from the stomach during the first 2 h of intestinal perfusion. The effect is very conspicuous when a high rate (5 ml/h) of intestinal perfusion was used, less so when the perfusion fluid was delivered at a lower rate. It is to be noticed that the decrease in effluent volume occurs irrespective of the degree of dilution of the perfusion SW. The experiments depicted in Fig. 3 were then continued for another 20 h, during which time PR was infused into the stomach. Then the fish were killed, BW estimated and blood samples drawn (Table I). For these initial PR experiments, no acid secretory data will be presented.

During the study the technique was changed, and C2 (instead of C2) was used to drain the stomach. This did not change the time-volume relationship (Fig. 2, lower panel, compare 'no injection C2' and 'no injection C2'), but was technically easier and decreased the number of experiments which had to be discarded due to malfunction of the gastric drainage. Neither did the use of C2 change the effect of intestinal perfusion on the rate of gastric volume effluence (Fig. 2). One series of fishes were intestinally perfused with 33% SW (high rate) between 22 and 24 h postsurgically. During that time the effluent volume decreased to zero in five of the eight fishes and to 0.18 ± 0.01 ml/h in the remaining three. In Fig. 2 (NaCl-injection C2) it is also shown that the i.m. injection of hypotonic saline (3.0 ml/h, 0.6% NaCl) depresses the rate of effluent volume output.

The values for the recovery of gastric perfusion fluid (Table I) provide additional information.

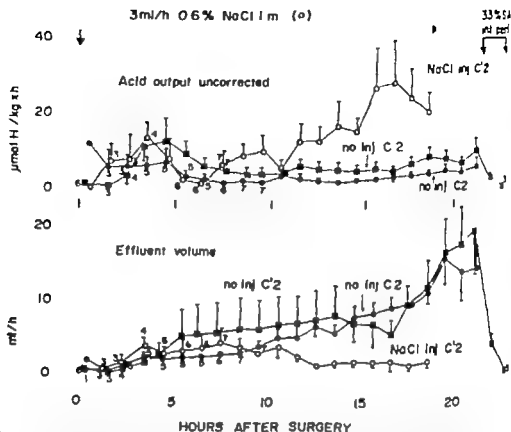


Fig. 2. Comparison of gastric acid secretion and gastric effluent volume for differently placed catheters to drain the stomach (C2 and C7). In one series, the fishes were intravenously perfused with 33% NaCl, during 2 h. The effect of i.m. saline is also shown. Each group comprised 8 animals; the number of fishes delivering any gastric effluence has been indicated when less than 8.

until assayed for osmolality (freezing point depression, Knauer osmometer), Na⁺ and K⁺-concentrations (Turner Model 510 Flame Photometer) and Cl⁻-concentration (Radiometer CMT 10 Chloride Titrator).

Drugs: Atropine sulfate, hexamethonium bromide, sodium salt of phenol red (Sigma), Metacaine (Kli o & French Laboratories), Heparine 5000 IE/ml (V-trum), MS-222 (Sandoz).

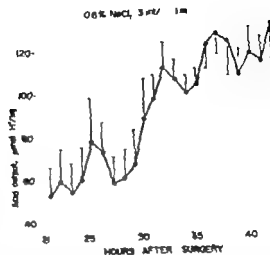
Statistics: Values in the Figures and the Table are mean and S.E. Group means were compared by a (Texas Instruments SR 52 calculator program ST1-07 two sample test, or ST1-06, paired observations) and linear regression calculated by the method of least squares (program ST1-08).

Results

1. Effluent volume

In previous reports (Holstein 1975, 1976, 1977) the term *drinking rate* was used to describe the hourly volume output from the stomach. Since that volume, at least theoretically, represents the sum of the positive terms *ingested water* and *volume secretion* from the stomach and a negative term representing *regurgitation* from the stomach, the term *effluent volume* although certainly in essence mirroring the ingested volume, will be preferred.

When starting this study the catheter used to drain the stomach, though slightly differently placed, was of the same type as used previously and the results confirm earlier observations. In experiments without perfusion of intestine or stomach, no gastric effluence or samples of very small volume were obtained for the first few hours following recovery from anaesthesia. Then the volume increased and finally stabilized at a level which varied from individual to individual (Fig. 2, lower panel, no injection C2).



4. Gastric acid secretion in fishes during the injection of hypotonic saline equivalent to consumption of one ration in Fig. 2 (NaCl 0.6%, C2). μ

on the rate of oral water ingestion. A value in excess of 100% indicates that fluid has been added to the perfusion solution. As losses of fluid through the oesophagus could mask this, the figures for mean recovery of perfusion volume have to be corrected for recovery of PR. Of course, even the corrected figures must be carefully interpreted as gastric volume secretion also is included in the effluent volume. It will be seen that for most groups, about 5% of the perfused volume (8.5 ml/h) was recovered, indicating that drinking was low absent. A notable exception is the group perfused with 30% SW at a low rate, for which the recovered volume was 37.6%. The second highest value, 130%, belongs to the Lm. saline injected group.

Gastric acid secretion

1. Secretion before perfusion of the stomach with PR (uncorrected values). In concert with previous findings, the secretion of gastric acid in pylorus ligated, non-perfused, non-injected fish was very low, not exceeding 10 μ mol H⁺/kg h. Two series of experiments are shown in Fig. 2, in which the stomachs were drained by C2 and C'2, respectively. In one series (C2), the fishes were killed at 22 h, their mean BW was then 90.5% of the initial value. In the other series (C'2), the experiment was terminated at 24 h, following two hrs of intentional perfusion with 33% SW. This shortlasting perfusion had no effect on the BW (90.9% of initial value), neither could any stimulation of the acid secretion be detected.

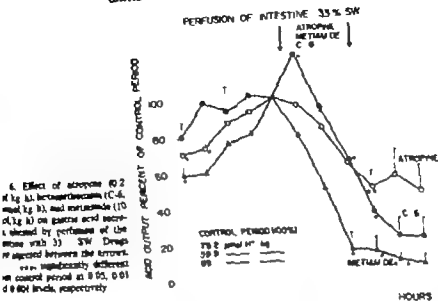
Fig. 2 also depicts the secretion of gastric acid in eight fishes Lm. injected with hypotonic saline from the start of the experiment. During saline injection the effluent volume was depressed, introducing a greater risk to underestimate the output of gastric acid (Holtekin 1977). Nevertheless, an increased acid secretion during saline injection is obvious. The injection rate was chosen to deliver a volume sufficient to compensate for the decrease in BW in 450 g fish with ligated pylorus. This rate (3 ml/h) was, however, used for all fishes irrespective of weight and was found to counteract the weight loss encountered without saline injection. When killed (23 h later) the experiment was continued with PR perfusion of the stomach, the mean BW was 103.1% of the initial value.

TABLE 1 Final body weight, recoveries of phenol red and gastric perfusion volume, and plasma parameters.

Fig.	Treatment	n	% BW	Recoveries		Plasma parameters				
				% PR	% Vol	% vol. 100 % PR	mOsm/l	Na (mM)	K (mM)	Cl ⁻ (mM)
2	no treatment	8	90.5 ± 0.9	—	—	—	384.5 ± 4.2	201.5 ± 3.8	5.3 ± 0.5	166.3 ± 2.5
2	int. perf 33 % last 2 h	8	90.9 ± 0.4	—	—	—	364.6 ± 4.1	199.2 ± 4.7	3.6 ± 0.1	172.8 ± 2.3
2 & 4	0.6 % NaCl Lm	8	103.1 ± 0.7	92.8 ± 2.1	120.8 ± 8.1	130.2	327.1 ± 2.9	190.0 ± 2.0	2.3 ± 0.1	152.4 ± 1.3
3	int. perf 100 % (high rate)	5	86.1 ± 1.8	72.8 ± 7.3	83.2 ± 7.0	114.3	530.3 ± 5.4	242.3 ± 12.7	5.6 ± 0.2	209.5 ± 11.0
3	int. perf 50 % (high rate)	5	92.9 ± 1.1	94.3 ± 1.6	103.4 ± 1.7	109.6	409.6 ± 8.7	192.8 ± 4.6	3.7 ± 0.2	175.0 ± 4.6
3	int. perf 90 % (low rate)	8	94.5 ± 0.7	99.3 ± 2.3	324.6 ± 83.8	326.9	396.7 ± 9.2	188.1 ± 3.1	3.3 ± 0.1	160.3 ± 2.3
3	int. perf 33 % (high rate)	5	101.2 ± 0.3	90.2 ± 5.0	98.9 ± 6.2	109.6	342.9 ± 9.8	153.6 ± 12.5	3.2 ± 0.3	162.8 ± 7.3
3	int. perf 33 % (low rate)	5	97.9 ± 1.0	—	100.4 ± 10.0	—	355.0 ± 13.2	172.2 ± 7.6	3.4 ± 0.1	157.6 ± 6.1
5	int. perf 100	6	82.6 ± 1.1	81.7 ± 4.6	93.3 ± 14.1	116.7	521.3 ± 14.3	254.8 ± 9.1	7.7 ± 0.5	217.0 ± 4.0
5	int. perf 67 %	4	83.4 ± 1.3	83.4 ± 1.3	90.4 ± 6.8	107.2	446.5 ± 17.2	191.8 ± 6.6	4.2 ± 0.5	221.3 ± 12.6
5	int. perf 50 %	8	100.8 ± 0.8	86.8 ± 2.1	96.5 ± 3.4	110.6	334.5 ± 4.3	183.5 ± 4.3	3.8 ± 0.2	153.6 ± 1.0
5	int. perf 33 %	10	103.3 ± 0.9	94.9 ± 1.9	113.0 ± 8.8	119.1	373.9 ± 3.3	179.9 ± 4.8	3.3 ± 0.3	156.9 ± 1.4
6	int. perf 33 % atropine	10	103.3 ± 0.6	88.7 ± 2.6	96.6 ± 2.9	103.8	360.4 ± 6.7	184.7 ± 6.8	3.0 ± 0.1	168.6 ± 5.1
6	int. perf 33 benzocaine	5	104.0 ± 1.2	86.0 ± 3.2	90.2 ± 3.1	104.8	362.9 ± 5.6	177.0 ± 2.4	2.9 ± 0.1	189.7 ± 6.1
6	int. perf 33 % metamido	6	102.0 ± 1.0	89.9 ± 6.0	96.2 ± 5.6	107.0	340.1 ± 9.1	173.6 ± 9.0	3.3 ± 0.2	152.0 ± 4.6

1 the experiments depicted. Fig. 3 perfusion of the intestine started on the day after surgery and PR 2 h later. 1 expts. shown. 1 Fig. 3-6 in ext. 1 perfusion started when the fishes had recovered from anaesthesia, 1 perfusion of the stomach with PR on the following d. y

GASTRIC ACID SECRETION IN TELEOST



V. Faecal BW: mean recoveries of gastric perfusion volume and PR, and plasma data appear in Table 1.

In a previous report (Hoback 1977), the effect of atropine on histamine-induced gastric acid secretion was described. Atropine, 2 or 5 μ mol/kg h was found to markedly depress the volume of gastric effluence, and the uncorrected output of gastric acid decreased. However when using PR (then injected at a rate of 0.62 ml/h) to correct for recovery no inhibition could be demonstrated. In the present work a higher rate of PR perfusion was employed (8.5 ml/h) and the dose-rate of atropine was lowered (0.2 μ mol/kg h). Despite these alterations the hourly effluent volume in three of the eight fishes fell during atropine administration and the recovery of PR decreased to minimally 52, 31.6 and 70% in the three fishes respectively. However following correction for recoveries the inhibition by atropine was still evident. Neither hexamethonium, nor methamidate in the doses employed, caused any apparent changes in the effluent volume output.

In Fig. 7 the group means for plasma osmolality sodium-chloride and potassium concentrations have been plotted against the corresponding group means for % BW. In all cases significant correlations were found. Values from three additional experiments in which the intestines were perfused with acidified SW or glucose (to be published separately) are included in Fig. 7.

Discussion

The present work has bearings on two problems, namely the regulation of gastric acid secretion and the regulation of oral intake of water in a marine teleost. The results strongly suggest that both are regulated by changes in the water balance.

In discussion, it will be supposed that a % BW below 100% indicates dehydration.

This was felt justified as the concentrations of plasma sodium, potassium, and also osmolality increased when the % BW decreased. It was found that treatment (perfusion of the intestine with 33% or 50% SW

PERFUSION OF INTESTINE

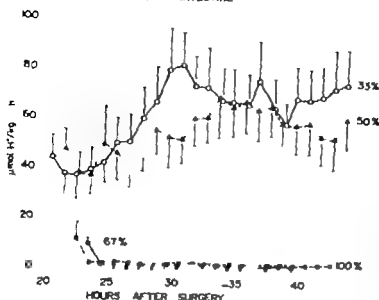


Fig. 5. Gastric acid secretion during perfusion of the intestine: pure sea water (100% SW), variously diluted SW. For details of animals, see Table I.

2.2. Secretion during perfusion of the stomach with PR (corrected values).

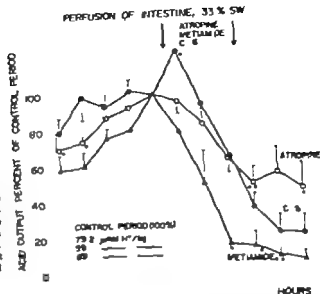
2.2.1 Injection of hypotonic saline The fishes used for this study had the C2 and C catheters placed only 3 ml/h of 0.6% NaCl was i.m. injected from the start, and PR perfusion of the stomach commenced at 20 h. Gastric acid secretion during PR is shown in Fig. 4 postexperimental BW (103.1%) recovers and blood data appear in Table I.

2.2.2. Perfusion of the intestine The results to be reported in this section concern fish equipped with catheters C1, C2 and C3. The intestinal perfusion (high rate) started immediately following recovery from anesthesia, and perfusion of the stomach with PR about 22 h postsurgically. In different experiments, the intestine was perfused with 100%, 67%, 50%, or 33% SW. Fig. 5 shows that the secretion was extremely depressed in fishes perfused with 100% or 67% SW, and Table I shows that these groups were unable to maintain their BW at 100%. Intestinal perfusion with 50% or 33% SW on the other hand, resulted in maintained BW and high rates of gastric secretion. The first fractions following the start of PR perfusion of the stomach contained more acid than the following; this is probably due to wash-out of acid accumulated during previous hrs.

When examining the fishes postexperimentally it was noticed that the intestines of fishes perfused with 33% or 50% SW were heavily loaded with water, while the 67% and 100% SW-perfused fishes had remarkably empty intestines. It should also be mentioned that additionally two fishes were prepared for the 100% SW experiment, but these fishes died following 32 and 36 h of perfusion and were discarded.

2.2.3 Effects of antagonists during intestinal perfusion The fishes prepared for these experiments had catheters C1, C2, and C3 positioned. The i.m. injection of the drug which started at different h postsurgically lasted for three hrs. Gastric acid secretion, corrected for PR recovery is presented as the percentage of the h preceding drug injection (Fig. 6). The absolute values for the respective control hour are given in the figure. Atropine ($0.2 \mu\text{mol/kg h}$), hexamethonium (C-6, $10 \mu\text{mol/kg h}$), and metiamide ($10 \mu\text{mol/kg h}$) all significantly inhibited the gastric acid secretion during perfusion of the intestine with 33%

Fig. 6. Effect of atropine (0.2 μ mol/kg h), betamethasone (C-4, 0 μ mol/kg h), and methiamide (M-4, 0 μ mol/kg h) on gastric acid secretion elicited by perfusion of the intestine with 33% SW. Drifts were rejected between the arrows. a, b, significantly different from control period at 0.05, 0.01 and 0.001 levels, respectively.



W. Final BW, mean recoveries of gastric perfusion volume and PR, and plasma data appear in Table I.

In a previous report (Holstern 1977), the effect of atropine on histamine-induced gastric acid secretion was described. Atropine, at 5 μ mol/kg h was found to markedly depress the volume of gastric effluence, and the uncorrected output of gastric acid decreased. However, when using PR (then ejected at a rate of 0.62 ml/h) to correct for recovery, no inhibition could be demonstrated. In the present work a higher rate of PR perfusion was employed (3.5 ml/h) and the dose-rates of atropine was lowered (0.2 μ mol/kg h). Despite these precautions the hourly effluent volume in three of the eight fishes fell during atropine administration, and the recovery of PR decreased to minimally 52, 31.6 and 20% in the three fishes respectively. However, following correction for recoveries the inhibition by atropine was still evident. Neither betamethasone, nor methiamide in the doses employed, caused any apparent changes in the effluent volume output.

In Fig. 7 the group means for plasma osmolarity, sodium, chloride, and potassium concentrations have been plotted against the corresponding group means for % BW. In all cases, significant correlations were found. Values from three additional experiments in which the intestines were perfused with acidified SW or glucose (to be published separately) are included in Fig. 7.

Discussion

The present work has bearings on two problems, namely the regulation of gastric acid secretion and the regulation of oral intake of water in a marine teleost. The results strongly suggest that both are regulated by changes in the water balance.

In the discussion, it will be supposed that BW below 100% indicates dehydration of the fish. This was felt justified as the concentrations of plasma sodium, potassium, and chloride and also osmolarity increased when the % BW decreased. It was found that treatments which prevented the dehydration (perfusion of the intestine with 33% or 50% SW

PERFUSION OF INTESTINE

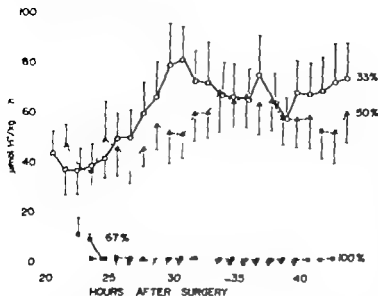


Fig. 5. Gastric acid secretion during perfusion of the intestine with pure sea water (100% SW) variously diluted SW. For number of animals, see Table I.

2.2. Secretion during perfusion of the stomach with PR (corrected values).

2.2.1 Injection of hypotonic saline The fishes used for this study had the C2 and C catheters placed only 3 ml/h of 0.6% NaCl was i.m. injected from the start, and PR perfusion of the stomach commenced at 20 h. Gastric acid secretion during PR is shown in Fig. 4 postexperimental BW (103.1%), recoveries and blood data appear in Table I.

2.2.2. Perfusion of the intestine The results to be reported in this section concern fish equipped with catheters C1, C2 and C3. The intestinal perfusion (high rate) started immediately following recovery from anesthesia, and perfusion of the stomach with PR about 22 h postsurgically. In different experiments, the intestine was perfused with 100%, 67%, 50%, or 33% SW. Fig. 5 shows that the secretion was extremely depressed in fishes perfused with 100% or 67% SW and Table I shows that these groups were unable to maintain the BW at 100%. Intestinal perfusion with 50% or 33% SW on the other hand resulted in maintained BW and high rates of gastric secretion. The first fractions following the start of PR perfusion of the stomach contained more acid than the following; this is probably due to wash-out of acid accumulated during previous hrs.

When examining the fishes postexperimentally it was noticed that the intestines of fishes perfused with 33% or 50% SW were heavily loaded with water while the 67% and 100% SW perfused fishes had remarkably empty intestines. It should also be mentioned that additionally two fishes were prepared for the 100% SW experiment, but these fishes died following 32 and 36 h of perfusion and were discarded.

2.2.3 Effects of antagonists during intestinal perfusion The fishes prepared for these experiments had catheters C1, C2, and C3 positioned. The i.m. injection of the drug which started at different h postsurgically lasted for three hrs. Gastric acid secretion, corrected for PR recovery is presented as the percentage of the h preceding drug injection (Fig. 6). The absolute values for the respective control hour are given in the figure. Atropine (0.2 $\mu\text{mol/kg h}$), hexamethonium (C-6, 10 $\mu\text{mol/kg h}$) and metiamide (10 $\mu\text{mol/kg h}$) all significantly inhibited the gastric acid secretion during perfusion of the intestine with 33% SW.

plasma (Skidhaug 1969). In the present work, the steady state concentration of the intestinal content during perfusion was not determined, and it is only possible to conclude that the turning point, during the present conditions, corresponds to a value below 67% SW probably between 50% and 55% SW.

The commencement of intestinal perfusion resulted in a rapidly decreasing rate of effluent volume output from the stomach. In some experiments (e.g. Fig. 3) there elapsed about 20 h between the observation on drinking rate and the sacrifice of the fish. It therefore remains with some uncertainty about the immediate effect of intestinal perfusion on the BW. However, in one series, the experiments were terminated following 2 h of intestinal perfusion with 50% SW. The final BW of this group ($90.9 \pm 0.4\%$) did not differ from that of the unperfused group ($90.5 \pm 0.9\%$). Thus, the initiation of 50% SW perfusion in previously unperfused fishes did not affect the BW within 2 h, i.e. the time period during which the effect of 'drinking' was observed. There was, however, a significant difference ($P < 0.01$) in the plasma osmolarity between the untreated group (384.5 ± 4.2 mOsm/l) and the group intraperitoneally perfused with 50% SW during the last 2 h (364.6 ± 4.1). Also plasma potassium is different between these groups ($P < 0.01$). That the decrease in any of these parameters suggests the interruption of oral water intake seems highly improbable, since the corresponding parameters for the group Lm. treated with hypotonic saline—which maintained a low rate of gastric volume output—exhibited still lower values. As 50% SW failed to affect the BW within 2 h, it seems highly unlikely that intestinal perfusion with more concentrated SW caused any initial, short-lasting increase in BW. Thus, in the case of 50% or 55% SW perfusion, 'drinking' was interrupted or decreased before any significant gain in BW had been achieved, while perfusion with 67% or 100% SW precipitated drinking without ever alleviating the 'thirst'.

The effect of intestinal perfusion on the rate of gastric volume effluence was independent of the degree of dilution of the perfusing SW but depended on the rate of perfusion. This suggests the participation of intestinal volume-sensitive receptors, the activation of which interrupts the intake of water despite the continuous drive from a diapaenetic factor (risk of dehydration). The conspicuous distension of the intestine following perfusion with 50% or 55% SW corroborates this interpretation, the equally conspicuous lack of fluid in the intestine following perfusion with 100% or 67% SW contradicts it. It must also be borne in mind that the effect on gastric effluent volume might be artifactual, elicited by mechanical deformation of the stomach due to distension of the intestine. This assumption is strengthened by the great amount of water found in intestines of 50% and 55% SW-perfused fishes but contradicted by the lack of water in intestines of fishes perfused with more concentrated SWs. The persistency of the effect, despite change of technique to drain the stomach (C2 to C3) also does not favour the artifact theory.

Referring to the preceding discussion, it seems unlikely that the depression of gastric effluent volume during Lm. injection of saline results from activation of the same mechanism which is responsible for the interruption of drinking when commencing intestinal perfusion. Speculatively the effect of Lm. saline is mediated by some change in plasma volume or composition, causing the withdrawal from the circulation of some factor stimulating water ingestion.

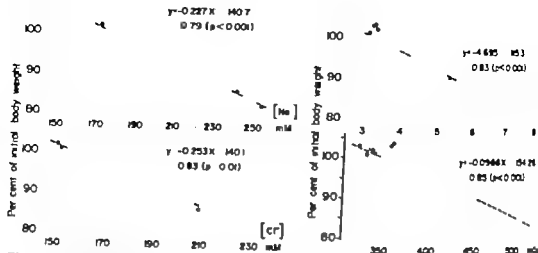


Fig. 7 Plots of different plasma parameters against final body weight expressed as a percentage of initial BW. The graphs include data from three experiments not reported in this communication (symbols).

LM injection of saline) precipitated or decreased the output of gastric effluence (drinking and increased the output of gastric acid. Perfusion with 67% or 100% SW neither maintained BW nor stimulated the gastric acid secretion, but 100% SW still precipitated drinking. No observations on gastric volume effluence were performed when starting intestinal perfusion with 67% SW but the mean recovery of the volume of PR pumped through the stomach amounted 107.2%, indicating that no significant oral intake of water took place. Concerning 50% SW it should be remarked that BW was maintained when the perfusion commenced as soon as the animals recovered from anesthesia (Fig. 5 and Table I) but when instituting the perfusion on the second day the fishes were unable to restore BW during the following 24 h (Fig. 3 and Table I). The results demonstrate that the Cod is unable to transport water from the intestine to the blood when the intestine is continuously perfused with 100% or 67% SW but easily do so when perfused with 33% SW. The fish is also able to obtain water from the intestine during perfusion with 50% SW but at a lower rate than when 33% SW is used. This interpretation closely agrees with results presented by Ueda and Hirano (1973), at least when making allowance for that in the present work continuous perfusion from a reservoir was used, while Ueda and Hirano measured water transport in intestinal sacs, isolated from SW eels. When the sacs were filled with 100% SW there was an increasing loss of water from the sacs during the experiment (5 h). When filled with 67% SW there was an initial loss of water but at about 3.5 h the flow reversed. With 50% SW a short-lasting, smaller loss was observed before the intestinal sacs gained water. When filled with 33% SW there was a fast net transport of water from serosa to mucosa during the whole experiment.

Also *in vitro* experiments in the eel confirm the present observations. Skadhauge (1967) perfused the intestine with 50% SW using a closed system where the water recirculated. Before any net resorption of water took place, there was an initial dilution of the perfusate fluid. The cited works indicate that sea water following entry of the teleostean intestine is diluted with endogenous water before any net resorption of water is possible. When the flow reverses (turning point) the osmolality of the luminal content is higher than that of

plasma (Skudhauge 1969). In the present work, the steady state concentration of the external content during perfusion was not determined, and it is only possible to conclude at the turning point, during the present conditions, corresponds to a value below 67% probably between 50% and 33% SW.

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that the effect on gastric effluent volume might be artifactual, elicited by mechanical de-

compression of the stomach due to distension of the intestine. This assumption is strengthened

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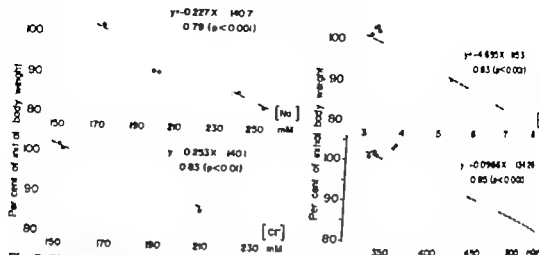


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acid secretion, but exerts slight or no influence on histamine or carbachol-induced acid secretion. (In light of the recent observation by Larsson and Rehfeld (1977), that gastrin and cholecystokinin can not be immunologically differentiated in the Cod, it might be better to substitute gastrin for gastrin-like peptide.) Furthermore, several attempts to demonstrate a vagal route in the regulation of acid secretion with the aid of insulin or 2-deoxy-D-glucose either failed (insulin) or gave inconsistent (2-DG) results (Cederberg and Hestén, unpublished). Provided there exists in the Codfish a vagal mechanism sensitive to hypoglycemia, these results further demand that the hypothetic antagonist should block also vagally induced acid secretion. From experiments with the isolated gastric mucosa from the

Rana temporaria, lysine vasopressin seems at least partially to fulfil these requirements. Simultaneous and pentagastrin-induced acid secretion was inhibited by ADH, while histamine, antipyrilene and dib-cAMP-induced secretion was not (Öbrink 1977). In the intact animal, however, where the interpretation is complicated by effects on gastric mucosal blood flow both inhibition and absence of inhibition by vasopressin (or pituitary extracts) in histamine-stimulated acid secretion in mammals have been reported (see review by Spiro and Britz 1972). In the fresh-water Codfish, pituitrin P^+ failed to inhibit acid secretion elicited by histamine, and by itself stimulated secretion (Gagzyan *et al.* 1968).

Vasopressin has been discussed because in the dehydrated Codfish a high titre of an antidiuretic principle would be expected. (Recall that antidiuresis could also be effected by increased renal renin activity.) However vasopressin has not been found in teleosts. Another peptide, arginine vasotocin, AVT is found in the teleostean hypothalamo-hypophyseal area (Sawyer 1977 for references), but its effects on gastric secretion has, to my knowledge, never been investigated. Although AVT has been reported to facilitate adaptation to SW in the flounder (Morris and Martz 1967), an increase in plasma AVT in the Codfish during dehydration is by no means certain. Injection of AVT in FW teleosts and lungfish produces diuretic effects (for references, see Pang 1977), but Henderson and Wales (1974) demonstrated diuretic effects of low doses, diuretic effects of high doses, in the eel. Lederis (1964) and a depletion of AVT in the neurohypophysis of Trout following exposure to SS (but Bentley (1971) failed to detect any difference in plasma AVT between FW and SS-adapted eels. For further discussion of the function of AVT in teleosts, is referred to Lederis (1964), Lacanillo (1972), and Pang (1977).

Although it is realized that extrapolations from mammalian physiology in attempts to place results obtained in a fish might be misleading, one more look at the mammals is in order. Several antestimal peptide hormones with more or less marked effects on gastric acid secretion have been described (see Baron 1976). Of these, bulbogastron seems to most closely fit the criterion given above for the inhibitory principle in the Codfish. Bulbogastron inhibits gastrin-, pentagastrin- and insulin-stimulated acid secretion. Also secretion induced by test meal and by sham feeding is inhibited, while histamine and urecholine induced secretion is not (Anderson *et al.* 1976). The stimulus for release of bulbogastron, distension of the duodenal bulb, however was not a condition prevailing in the Codfish. Future work, aiming at the disclosure of the inhibitory factor sympathetic nervous activity correlating catecholamines should also be considered (*cf.* Sanders 1976). Whatever the mechanism, the inhibition of gastric acid secretion in states of dehydration is probably a

In mammals, angiotensin II (AII) has been suggested to be a dipsogenic factor (Fitz 1969 1975) and is released in states of dehydration (Abdelal *et al* 1976) and following haemorrhage (Russel *et al* 1975). Such a function also in the teleost is purely speculative but in the eel plasma renin activity (Sokabe and Nakajima 1972, Henderson *et al* 1975) and drinking (Orde and Utida 1968) increases following transfer of fresh water (FW) fish to SW. The renin content of the kidney is lower in FW species than in SW (Miyogami *et al* 1968) and decreases following transfer of FW-eels to SW (Sokabe 1968). These authors suggest that renin-angiotensin regulates glomerular filtration rate in teleosts so that urine production decreases in SW. Sokabe and Nakajima (1972) postulate that the increased plasma renin activity in SW may be the cause of the increased water reabsorption in fishes following transfer to SW. All these effects would be of survival value also for fishes during pyloric ligation and ensuing water deprivation. As the list of hormones known or suspected to participate in the adaptation of euryhaline fishes to SW is long (for references, see Johnson 1973) there are several other candidates for a dipsogenic factor. Further speculations will be refrained from; it should only be mentioned that the dipsogenic factor seems to be of extra hypophysial origin, since Gaitakelli and Chester Jones (1971) found that hypophysectomy had no effect on the drinking rate in SW eels.

Perfusion of the intestine also elevated the output of gastric acid, provided the perfusion resulted in a maintained BW. When the perfusion was initiated on the second day post-surgically effluent volume decreased rapidly (Fig. 3) but there elapsed 5–10 h before any sign of stimulated acid secretion was detectable (not illustrated). This indicates that the secretory effect, in contrast to the effect on effluent volume, was mediated by a change in composition or volume of the extracellular fluid. I.m. injection of hypotonic saline, at a rate allowing the maintenance of BW, also was accompanied by a high output of gastric acid. It can not be excluded that the elevated acid output accompanying saline injection is the one hand and intestinal perfusion on the other results from different mechanisms. It seems more likely and is tentatively suggested that a common mechanism—acting on extracellular composition or volume—was called into action. The high output of gastric acid could result from the release of a stimulating factor from the withdrawal of an inhibitory factor or from a combination of both. The most straightforward interpretation of the results is that dehydration triggers the release of some factor(s) which stimulates the intake of water and inhibits gastric acid secretion. Whether the same or different factors mediate both effects can not be decided at the present time. Some characteristics of the secretory inhibitor can, however, be deduced from earlier works (Holstein 1975 1976). It was found that in pylorus-ligated fishes, without any water substitution, gastric acid secretion was very low in the basal state but could be stimulated by histamine and carbachol but not by pentagastrin. Also bombesin failed to elicit gastric secretion in these fish (Holstein, unpublished observation). Bombesin, a peptide from frog skin, in mammals elicits gastric acid secretion by release of endogenous gastrin (Basso *et al* 1974, 1975, Bertolini *et al* 1974, Impicciatore *et al* 1974). Due to these previous results, it was concluded that gastrin either is not present in the Codfish, or present but not involved in the regulation of gastric acid secretion. The present results offer the alternative interpretation, that in water-deprived fishes there circulates a factor which potentially inhibits basal and gastric

acid secretion, but exerts slight or no influence on histamine or carbachol-induced acid secretion. (In light of the recent observation by Larsson and Rehfeld (1977), that gastrin and cholecystokinin can not be immunologically differentiated in the Cod, it might be better to substitute gastrin for gastrin-like peptide.) Furthermore, several attempts to demonstrate a vagal route in the regulation of acid secretion with the aid of insulin or 2-deoxy-D-glucose either failed (insulin) or gave inconsistent (2-DG) results (Cederberg and Olsson, unpublished). Provided there exists in the Codfish a vagal mechanism sensitive to polypeptides, these results further demand that the hypothetic antagonist should block also vagally induced acid secretion. From experiments with the isolated gastric mucosa from the eel *Rana temporaria*, lysine vasopressin seems at least partially to fulfil these requirements, since histamine- and pentagastrin-induced acid secretion was inhibited by ADH, while histamine- and dibutyryl-cAMP-induced secretion was not (Öbrink 1977). In the intact animal, however, where the interpretation is complicated by effects on gastric mucosal blood flow both inhibition and absence of inhibition by vasopressin (or pituitary extracts) on histamine-stimulated acid secretion in mammals have been reported (see review by Gero and Britz 1972). In the fresh-water Catfish, pituitrin P^+ failed to inhibit acid secretion elicited by histamine, and by itself stimulated secretion (Gritzman *et al.* 1968). Vasopressin has been discussed because in the dehydrated Codfish a high titre of an antidiuretic principle would be expected. (Recall that antidiuresis could also be effected by raised renal renin activity.) However, vasopressin has not been found in teleosts. Another peptide, arginine vasotocin, AVT is found in the teleostean hypothalamo-hypophyseal axis (Sawyer 1977 for references), but its effects on gastric secretion has, to my knowledge, never been investigated. Although AVT has been reported to facilitate adaptation to SW in the Gouander (Molina and Maetz 1967), an increase in plasma AVT in the Codfish during dehydration is by no means certain. Injection of AVT in FW teleosts and lungfish produces diuresis (for references, see Pang 1977), but Henderson and Wales (1974) demonstrated diuretic effects of low doses, diuretic effects of high doses, in the eel. Lederis (1964) and a depletion of AVT in the neurohypophysis of Trout following exposure to 85% V but Bentley (1971) failed to detect any difference in plasma AVT between FW and V-adapted eels. For further discussion of the function of AVT in teleosts, is referred to Maetz (1968), Lacaille (1972), and Pang (1977).

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physiological mechanism of value for an animal living in, and forced to drink of a hypotonic medium.

If the high rate of acid secretion in the fishes which maintained their BW can be explained by the absence of an inhibitory factor the problem remains to find the agonist. Before subjected to experimentation, the fishes were starved for at least one week. Therefore, hypoglycemic secretion is possible. Experiments on vagotomized animals should help to clarify this point, and radioimmunologic methods should be employed to study the possible involvement of a peptide hormone. The experiments with antagonists (Fig. 6) offer sufficient information to determine the nature of the secretory stimulus. As metiamide is not atropine inhibits histamine induced acid secretion in the Codfish (Holstein 1976, 1977) it is only possible to exclude this amine from the list of possible agonists. The inhibitory effect of atropine and hexamethonium suggests the participation of a nervous component although the selectivity of hexamethonium as a ganglionic blocker must be interpreted with some care (Stenvenson and Grove 1977).

Finally the present results indicate that the basal acid secretion in the codfish is somewhat higher than was previously suggested (Holstein 1975). It is interesting that the pH (determined with a two-indicator paper) of the water film present on the gastric mucosa of starved Codfishes killed for other purposes was 2.1 ± 0.7 $n = 15$ (Blycher and Holstein, unpublished observations).

I am indebted to Mrs Inga Maj Örbom for expert technical assistance to Mr Ingemar Hakar for supplying fish, and to Smith, Kline & French Labs. for a gift of metiamide. The work was supported by grants from Magnus Bergwall's Stiftelse and from Wilhelm och Martina Lundgrens Vårskapsfond.

References

- ABDELAAL, A. E., P. F. MERCER and G. J. MOGENSEN, Plasma angiotensin II levels and water intake following β -adrenergic stimulation, hypovolemia, cellular dehydration and water deprivation. *Pharmacol. Biochem. Behav.* 1976. 4: 317-321.
- ANDERSSON, S., G. NILSSON and L. SÖDDIN, The significance of the duodenal bulb for gastric function in the dog. *Scand. J. Gastroenterol.* 1976. 11 (Suppl. 42): 49-54.
- BARON, J. H., Inhibition of gastric secretion by intestinal hormones. *Scand. J. Gastroenterol.* 1976. 11 (Suppl. 42): 17-22.
- BASSO, N., G. IMPROTA, P. MELCHIORRI and N. SOPRANZI, Gastrin release by bombesin in the acid peptic dog. *Rivista Gastroenterol.* 1974. 6: 93-98.
- BASSO, N., E. LEZOCHE, A. M. TERIA, S. GILI and V. SPERANZA, Effect of bombesin on extragastric gastric secretion. *Amer. J. dig. Dis.* 1975. 20: 923-927.
- BENTLEY, P. J., Failure to detect levels of vasotocin similar to those in amphibians in the plasma of the eel, *Anguilla rostrata*. *J. Endocr.* 1971. 49: 183-184.
- BERTACCINI, G., V. ERSPAMER, P. MELCHIORRI and N. SOPRANZI, Gastrin release by bombesin in the rat. *Brit. J. Pharmacol.* 1974. 52: 219-225.
- FITZGERALD, J. T., The role of a renal thirst factor in drinking induced by extracellular stimuli. *J. Physiol. (Lond.)* 1969. 201: 349-368.
- FITZGERALD, J. T., Endocrine mechanisms in the control of water intake. In: *Neural integration of physiological mechanisms and behaviour*. Eds. MOGENSEN G. J. and F. R. CALAREPU. University of Toronto Press, Toronto. 1975. 226-247.
- GUTERELL, R. E. and I. CHESTER JONES, Drinking and urine production in the European eel (*Anguilla anguilla* L.). *Gen. comp. Endocr.* 1971. 16: 478-483.
- GROZYAN, D. M., M. G. ZAKS and O. P. TANAYEVICH, Effect of histamine and pituitrin on gastric secretion of the European catfish (*Silurus glanis* L.). *Problems of Acetylcholine* 1968. 8: 97-100. (Translation from *Vopr. Ikhtiol.* 1968. 8: 126-130.)

- MOORE, L. W. and N. A. M. WALKER, Renal diuretic and antidiuretic after injection of arginine vasotocin in the freshwater Eel (*Anguilla anguilla* L.). *J. Endocr.* 1974. 61 487-500.
- MOORE, L. W., V. JOTIMAKKALA, W. MOUNT and M. OJURI, Endocrine and environmental influences on plasma cortisol concentrations and plasma renin activity of the Eel, *Anguilla anguilla* L. *J. Endocr.* 1976. 70. 81-95.
- LITTEY, B., Gastric acid secretion in teleostean fish. A method for the continuous collection of gastric effluence from swimming fish and its response to histamine and pentagastrin. *Acta physiol. scand.* 1975. 5 417-423.
- LITTEY, B. Effect of the H_2 -receptor antagonist metiamide on carbachol- and histamine-induced gastric acid secretion in the Atlantic cod, *Gadus morhua*. *Acta physiol. scand.* 1976. 97 189-195.
- LITTEY, B. Effect of atropine and EC 15396 on stimulated gastric acid secretion in the Atlantic cod, *Gadus morhua*. *Acta physiol. scand.* 1977. 101 185-193.
- FRACCIATORE, M., M. CHIA ARNO, E. MOLINA and G. BEN ACCOPI, Osservazioni precliniche sul meccanismo d'azione della bombesina sulla secrezione gastrica del cane. *Atenee Farmaceut.* 1973. 44 15-20.
- FRANK, D. W. Endocrine control of hydromineral balance in teleosts. *Amer. Zool.* 1973. 13. 799-818.
- CANALAS, F. The trophic hydromineral factor of fishes II: Chromatographic and pharmacologic indications of similarity to arginine vasotocin. *Gen. comp. Endocr.* 1972. 19 413-420.
- FRANK, L.-L. and J. F. REYNOLDS, Evidence for common evolutionary origins of gastrin and cholecystokinin. *Nature (Lond.)* 1977. 269 335-338.
- TEDES, K., Free structure and hormone content of the hypothalamo-neurohypophyseal system of the Rainbow trout (*Salmo gairdneri*) exposed to sea water. *Gen. comp. Endocr.* 1964. 4 638-661.
- MATZ, J. Salt and water metabolism. In: *Physiological and endocrinology*. Eds. BARRINGTON, E. J. W. and C. BARKER ROBINSON. Academic Press 1968. 47-162.
- FRANK, S., M. OJURI, H. OKADA and H. NISHIMURA, Presence of renin in the glomerular and glomerular kidney of marine teleosts. *Amer. J. Physiol.* 1968. 215 991-994.
- STILL, R. and J. MATZ, Arginine vasotocin et évolution de la perméabilité branchiale au sodium au cours du passage d'eau douce en eau de mer chez le Flét. *J. Physiol. (Paris)* 1967. 59 271.
- FRANK, E. J. Studies on the kinetics of the peristaltic secretions of the stomach. *Acta physiol. scand.* 1948. 6/3 Suppl. 51 1-106.
- FRANK, E. J. ADH as an inhibitor of gastric acid secretion. In: *Gastric ion transport* (Abstracts from symposium held in Uppsala 1977). Uppsala 1977 102.
- DE, H. and S. UEDA, Changes in intestinal absorption and renal excretion of water during adaptation to sea-water in the Japanese eel. *Marine Biol.* 1968. 1 172-177.
- NG, P. T. Osmoregulatory functions of neurohypophyseal hormones in fishes and teleosts. *Amer. Zool.* 1977. 17 739-749.
- WILL, P. J. D., A. E. ASHCROFT and G. J. MCGOWAN, Graded levels of haemorrhage, thirst and arginine II in the rat. *Physiol. Behav.* 1973. 13 117-119.
- NOEL, D. J. A review: The effects and actions of sympathetic nerve and catecholamine inhibition of gastric secretory function. *Agents and Actions* 1976. 6. 383-388.
- FRANK, W. H. Evolution of neurohypophyseal principles among the vertebrates. *Amer. Zool.* 1977. 17 737-777.
- FRANK, R. and L. G. BERRY, The action of vasopressin on the gastro-intestinal tract. A review of the literature. *Amer. J. Dig. Dis.* 1972. 17 649-667.
- ALANCAUX, E., The mechanism of salt and water absorption in the intestine of the Eel (*Anguilla anguilla*) adapted to waters of various salinities. *J. Physiol. (Lond.)* 1969. 204 135-158.
- MATZ, H. W. The absorption and excretion of water and salts by marine teleosts. *Amer. J. Physiol.* 1970. 43 480-505.
- OKADA, H. and T. NAKAMURA, Chemical structure and role of angiotensin in the vertebrates. *Gen. comp. Endocr.* 1972. Suppl. 3 382-392.
- OKADA, H., S. MIYAGAKI and A. SATO, Role of renin in adaptation to sea water in euryhaline fishes. *Jap. J. Pharmacol.* 1968. 18. 332-343.
- FRANK, E. V. and D. J. GROVE, The extreme innervation of the stomach of the Fluke, *Physostomus physostomus* L. — I The vagal nerve supply. *Comp. Biochem. Physiol.* 1977. 38C. 143-151.
- STILL, R. and T. HENDON, Effects of changes in environmental salinity on salt and water movements in the intestine and gills of the Eel, *Anguilla japonica*. In: *Responses of fish to environmental changes*, Ed. C. H. W. C. C. Thomas Publisher Springfield 1973. 240-269.

Synthesis of ^3H acetylcholine in the rabbit lacrimal gland and its release by electrical field stimulation

By

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Abstract

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Electrical field stimulation was applied to the rabbit lacrimal gland during incubation in ^3H -acetylcholine, after which the flux of tritiated metabolites was studied. In the presence of physostigmine ^3H -acetylcholine was released into the incubation medium when the gland was stimulated electrically. The identity of the ^3H -acetylcholine synthesized by the preparation was established by its identical ion exchange chromatographic properties to natural acetylcholine. The tissue contents of ^3H -acetylcholine and ^3H -choline were 14.4 and 3.6%, respectively of the total activities found in the gland. This study demonstrates that the rabbit lacrimal gland is innervated cholinergically. It is suggested that the method developed might have value for studying effects of drugs on cholinergic neurotransmission in this organ.

The main secretory innervation of the mammalian lacrimal gland is thought to be cholinergic (Dementschenko 1872, Teplachine 1894, Aronson and Wilson 1971). In previous studies the cholinergic neurotransmission in this organ measurement of tear secretion has generally been used for indirect determination of acetylcholine secretion from the nerves. Recently a radiochemical method for studying acetylcholine release from isolated guinea pig liver was described (Wikberg 1977). In the present work a similar approach was used to study the release of acetylcholine from isolated rabbit lacrimal glands. Electrical field stimulation was applied to the organ during incubation with ^3H -choline whereafter the release of ^3H -acetylcholine synthesized by the organ could be examined.

Methods

Rabbits of either sex were killed by blow on the neck and exsanguinated. One of the lacrimal glands was rapidly excised through the caudal supraorbital notch and its capsule was carefully dissected away with forceps. The organ (weighing 114 ± 11 mg), was mounted in a 4 ml water-jacketed perspex chamber containing Krebs solution (Wikberg 1977) with eserine sulphate $3 \cdot 10^{-6}$ M. The solution was kept at 37°C and gassed with a mixture of O_2 (95%) and CO_2 (5%).

The lacrimal gland was incubated with $9.3 \cdot 10^6$ Bq (becquerel, dps) of ^3H -choline (methyl ^3H -choline chloride $3.7 \cdot 10^4$ Bq/nmole, Radiochemical Centre, Amersham, England) at a final concentration of $1 \cdot 10^{-6}$ M for 1 h. During the incubation the organ was stimulated electrically by 0.1 ms, 320 mA



1. Release of total metabolites from the lacrimal gland. After incubation with H-choline the organ was washed for 45 min before any fractions were collected. Release of acetylcholine (●—●), H-choline (○—○) and total (▲—▲) are presented. The graphs show the mean \pm of 5 experiments.

stimulus at 0.1 Hz by means of two 20 \times 1 mm platinum electrodes separated 10 mm apart on each side of the organ, using Grass SD9 stimulator and home-constructed unit. After the incubation (the electric stimulation was stopped) and the organ was washed with Krebs solution at intervals of 5 min for 45 min, after which 4 ml fractions were collected for analysis.

Electrophoresis

Method described previously (Walberg 1977), with some minor modifications, was used, to separate radioactivity of the samples into 3 fractions: 3 H-acetylcholine, H-choline and fractions which remained precipitated by ammonium molybdate (UPF). In order to improve the paper chromatographic separation of acetylcholine and choline, descending rather than the ascending system with acetone/water/10 N NH_4OH 20:20:1 (by volume) as used. The chromatograms were allowed to run for 18 h, using the standard technique. To facilitate the identification of carrier choline and acetylcholine the spots were developed by spraying with an iodophosphoric reagent (Walicki *et al.* 1967). By this method the absolute distances between the spots was 3 cm. In order to elute the radioactivity from the paper and to decolorize the iodophosphoric reagent, a mixture of 3 ml ethanol/0.5 M NH_4OH 1:1 (v/v) was used. These samples could be counted in addition of benzene (Packard Instruments Company Inc.) as described previously.

After the experiment the lacrimal gland was frozen in Prigen solid CO_2 and kept at -80°C until analysed. A tissue was homogenized with glass homogenizer in 0.4 M HClO_4 (1 + 0.5 ml) at 0°C and then centrifuged at 1000 g for 30 min. The pellet was washed once with 1 ml of the HClO_4 solution. The pooled supernatant was diluted to 25 ml and 1 ml fractions were taken for analysis as described above for the other solutions. The pellet was dissolved by adding 2 ml Soluene 350 (Packard Instruments Company Inc.) and counted in Packard Tri-Carb 3375 liquid scintillation spectrometer after addition of toluene and scintillator.

Thin layer chromatography

Approach similar to that described previously was used (Walberg 1977). Columns measuring 4.4 \times 430 mm were packed with Amberlite CG 90 II (200–400 mesh) equilibrated with 0.1 M sodium phosphate buffer pH 7.0. The samples applied on the columns were eluted with 5 ml H_2O followed by the phosphate buffer at a flow rate of 5 ml/min. In order to discriminate the retention volume of acetylcholine, 1.3 \times 10^6 Bq 3 H-acetylcholine (Acetyl-L- 3 H-acetylcholine iodide 8.5×10^6 Bq/mmol, NEN) was added to the sample and the radioactivity was separated from the tritium activity by measurement at separate channels in the

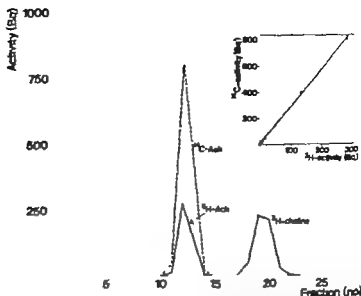


Fig. 2. Ion exchange chromatography of the reineckate-precipitated fraction in the washing solution of the lacrimal gland. The medium was collected during electrical field stimulation at 10 Hz and the quaternary ammonium compounds were precipitated by addition of manganous-reineckate in the presence of acetylcholine and choline. After batch treatment with Biorex 90 water and addition of 15 μ l 14 C-acetylcholine as a marker, the sample was applied on the column, followed by elution with 5 ml and 0.1 M phosphate buffer pH 7.0. Insert: Correlation between the 3 H-activity in the first peak and 14 C-activity of the 14 C-acetylcholine marker ($r = 1.000$).

Liquid scintillation spectrometer. The data obtained were corrected for quenching and for overlap between channels, using an internal standard and a double isotope computer programme written for IBM. The counting efficiency was 15% for 3 H and 51% for 14 C.

Results

During unstimulated conditions almost no tritiated acetylcholine was released from organ. After the 45-min washing period when collection of fractions was started, only 10% of the radioactivity released to the bathing solution consisted of 3 H-acetylcholine. No other activity at this time consisted of UPF (79%), followed by 3 H-choline (19%). Electrical field stimulation with 10 ms pulses at 10 Hz induced a 21-fold increase in the release of acetylcholine. The release of acetylcholine was not maintained at that level but decreased to half its maximal value in approximately 10 min, despite continuous electrical stimulation. The electrical stimulation also induced a two-fold increase in the release of 3 H-choline; during the subsequent stimulation periods this release showed a smaller tendency to decrease than that of 3 H-acetylcholine. The release of UPF also increased approximately 2-fold after electrical stimulation (Fig. 1).

The identity of 3 H-acetylcholine released during electrical stimulation was verified by ion exchange chromatography. After application of the reineckate precipitated fraction on column two peaks of radioactivity appeared. The first peak showed excellent correlation to the marker 14 C-acetylcholine peak (correlation coefficient 1.000) (Fig. 2).

1. Trained metabolites present in the lacrimal gland at the end of the expt. The radioactivity was separated into 4 fractions: the ammonium molybdate unprecipitated fraction (UPF), acetylcholine (Ach), choline (Chol) and the perchloric acid insoluble fraction (Pellet). The table gives the mean \pm S.E. of 5 expts.

10^{-4} Bq

7.55 ± 1.02	38.1
2.85 ± 0.35	14.4
0.72 ± 0.07	3.6
8.68 ± 1.44	43.8

When the expt. started the tissue content of tritium was $2.18 \pm 0.21 \cdot 10^4$ Bq. This amount responded to 23.4% of the total activity of ^3H -choline added to the incubation medium at the start of the expt. During the course of the expt. a total of $1.99 \pm 0.60 \cdot 10^4$ Bq of ^3H was released from the lacrimal gland. The secretion of ^3H -acetylcholine was $1.22 \pm 0.10 \cdot 10^4$ Bq, of ^3H -choline $2.93 \pm 0.30 \cdot 10^4$ Bq and of UPF $1.64 \pm 0.77 \cdot 10^4$ Bq. At the end of the expt. a considerable amount of ^3H -acetylcholine was present in the tissue but the choline content was low. The largest amounts of activity however were found as UPF in the PCA-insoluble fraction (Table I).

Discussion

The innervation of the mammalian lacrimal gland has been studied by histological techniques in several earlier studies. Ichikawa and Nakajima (1962) showed that the lacrimal gland of the rat contains unmyelinated nerve fibres in the inter- and intra-tubular connective tissues. Electron microscopic studies on lacrimal glands of monkeys have indicated that parasympathetic nerve fibres originating in the sphenopalatine ganglion innervate interstitial and parenchymal tissues (Ruskeff 1969). The arteriole of the monkey lacrimal gland may receive both cholinergic and sympathetic nerves (Ruskeff 1967-1969). Histochemical staining for cholinesterase in the human lacrimal gland has revealed choline esterase activity in myoepithelial cells and nerve fibres surrounding the acinar cells (Mizukawa *et al* 1962).

The present study demonstrates that the rabbit lacrimal gland is capable of accumulating tritium when incubated with ^3H -choline. It may be estimated that a passive uptake of ^3H of about 5% of the amount in the incubation medium will take place under the experimental conditions used. The high uptake observed (23.4% of the given amount) therefore suggests that an active mechanism for choline transport into the tissue is present. This transport system could be located in nervous tissue as well as in glandular cells. Active uptake of choline into nervous tissue is well documented (Pert and Snyder 1974, Simon *et al* 1976, Wikberg 1977), but other cells such as human red blood corpuscles may have transport systems for choline accumulation (Clement and Colthoun 1975).

In this work it was found that the rabbit lacrimal gland is capable of synthesizing ^3H -acetylcholine from ^3H -choline. The chemical identity of ^3H -acetylcholine seems to be certain, since the metabolite showed properties identical to those of native acetylcholine in different chromatographic systems. The basal release of ^3H -acetylcholine into the in-

cubation medium was almost negligible. This contrasted markedly with the results obtained from guinea pig ileum (Wikberg 1977) where the unstimulated release was much higher and showed spontaneous fluctuations. Electrical stimulation with stimulation parameters indicating that selective stimulation of nervous tissue was induced, resulted in a large increase in the release of H-acetylcholine from the lacrimal gland. The results of these experiments may be taken as fairly clear evidence of the presence of cholinergic innervation in this tissue.

The tissue content of H-acetylcholine was more than 20-fold larger than the amount of H-acetylcholine released during the electrical stimulation. In spite of this the release of H-acetylcholine declined rapidly after the initial large release. This observation might indicate that different compartments of acetylcholine are stored in the nerves, with variable capacity to release upon stimulation. Collier and MacIntosh (1969) have suggested that the acetylcholine turn-over in the superior cervical ganglion in cats takes place in non-uniform pools and that the newly synthesized acetylcholine shows a preferential release upon preganglionic stimulation. An alternative explanation for the decline of the release observed in this study may be that a regulatory mechanism is present which after a time diminishes the release of acetylcholine.

Besides H-acetylcholine, H-choline was also released into the incubation medium. Although a rather long washing period (45 min) had been used before any fractions were collected it is probable that the H-choline was derived predominantly from intracellular sources. The tissue content of H-choline at the end of the experiment was rather low, however this might indicate that H-choline was derived from some other kind of radioactive metabolite. It seems improbable that the H-acetylcholine released was responsible for the H-choline formation, since a rather high concentration of acetylcholine-esterase inhibitor was used and the release of the two metabolites showed a poor temporal correlation (Fig. 1).

Other metabolites were also present in the washing medium and in the tissue. This study gives no definite information on the nature of UPF. It might represent such metabolites as phosphorylcholine, betaine or N,N-dimethylethanolamine. It has been suggested that phosphorylcholine and H-betaine may be synthesized from H-choline in a variety of tissues (Sung and Johnstone 1965; Collier and Lang 1969; Szerb 1975).

In conclusion, it is proposed that the use of H-choline for labelling of intrinsic acetylcholine stores with tritium might be of value for studying acetylcholine release from the lacrimal gland. Recently this approach was used to investigate the influence of β -adrenoceptor antagonists on the cholinergic transmission in the rabbit lacrimal gland (Åberg *et al.* 1978).

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References

- ÅBERG, G. G. ADLER and J. WIKBERG, Inhibition and facilitation of lacrimal flow by beta-adrenergic drugs. *Acta ophthalmol.* (Kbh.), In press.
- CLEMENT, J. G. and E. H. COLHOUN, Inhibition of choline transport into human erythrocytes by diethylmethylaziridinium ions. *Canad. J. Physiol. Pharmacol.* 1975 53 1089-1093.
- COLLIER, B. and C. LANG, The metabolism of choline by a sympathetic ganglion. *Canad. J. Physiol. Pharmacol.* 1969 47 119-121.

- CLINE, B. and F. C. MACINTOSH, The source for acetylcholine synthesis in sympathetic ganglion. *Canad. J. Physiol. Pharmacol.* 1968, 47, 127-133.
- ERDMAN, J. Zur Innervation des Thrillastrobilus. *Pflügers Arch. ges. Physiol.* 1872, 7, 191-200.
- IKAWA, A. and Y. NAKAJIMA, Electron microscope study on the lacrimal gland of the rat. *Jap. J. exp. Med.* 1962, 77, 136-149.
- IKAWA, T., T. OYORI, J. HARA and M. IGA, Histochemistry of the human lacrimal gland. *Jap. J. Ophthalmol.* 1962, 6, 17-23.
- LEE, C. B. and E. H. BRYDER, High affinity transport of choline into the sympathetic plexus of guinea-pig. *Neuroscience J. Pharmacol. exp. Ther.* 1974, 191, 102-108.
- LYNN, J. R., E. ATWEK and M. J. KURON, Sodium dependent high affinity choline uptake: A regulatory step in the synthesis of acetylcholine. *J. Neurochemistry* 1976, 26, 909-921.
- MAH, C.-D. and R. M. JORDANOV, Evidence for active transport of choline in rat kidney cortex slices. *Canad. J. Biochemistry* 1963, 41, 1111-1118.
- JOE, J. C., Endogenous acetylcholine release and labelled acetylcholine formation from (³H)-choline in the sympathetic plexus of the guinea pig thorax. *Canad. J. Physiol. Pharmacol.* 1975, 53, 566-574.
- RELIACINE, A. Recherches sur les nerfs sécrétoires de la glande lacrymale. *Arch. ophthal. (Paris)* 1894, 14, 391-413.
- WALACE, M. B., A. M. GOLDENRO and F. E. SANDERSON, The synthesis of labelled acetylcholine by the rat isolated cat heart and its release by vagal stimulation. *Int. J. Neuropharmacol.* 1967, 6, 317-323.
- WILKINSON, L., Release of ³H-acetylcholine from isolated guinea pig ileum. A radiochemical method for studying the release of the cholinergic neurotransmitter in the intestine. *Acta pharmacol. scand.* 1977, 101, 302-317.

A healing promoting factor in rat wound fluid

By

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The origin and nature of stimulus to cells and tissues to repair after an injury is incomplete known. The stimulation for wound-healing may be due to a positive, healing-promoting factor. Such substances may be formed and released either systemically or locally. The aim of the present study was to examine whether a healing-promoting factor is formed or released, and, in the former case, if it increases the tensile strength. This may be used as a mechanical indicator of the extent of the repair of the tissue injured (Howes, Sooy and Harvey 1951; Sandblom, Petersen and Muren 1953).

Fluid produced in a stainless wire mesh cylinder subcutaneously implanted, is called interstitial wound fluid (Schilling, Joel and Shurley 1959; Hunt *et al.* 1967). If a stimulating factor is liberated locally it may exist in this fluid. The tensile strength of wounds to which such fluid was added, was determined. An increase would indicate the presence of a healing promoting factor.

The formation of granulation tissue of the healing wound is considered to increase when a stimulating factor exists. Therefore, cellulose sponges were implanted in wounds to test this effect assuming that increases in weight are due to stimulation of connective tissue formation (Grindlay and Weng 1961).

Rabbits weighing about 3 kg were used. The animals were anesthetized with Nebumal® (30 mg/kg). Two incisions about 2 cm long were made on each side of the middle line of the back. Through each incision a stainless wire mesh cylinder (5 cm long and 2 cm in diameter) was implanted s.c. The incision was closed by single silk suture (3/0). One week later the cylinder was punctured and the fluid collected and called wound fluid. Serum was received by puncture of ear veins of rabbits.

3 longitudinal skin incisions of a length of 4 cm and reaching underlying muscular fascia were made on each side of the back of rabbits. The distance of 2.5 cm from the midline. 2 ml of serum was added to the wounds on one side, and 2 ml of the wound fluid to the contralateral wounds. The wounds were closed by 3/0 silk.

The tensile strength of the wounds were determined by the method described by Sandblom *et al.* (1953) one week after the incision. The rabbit was anesthetized and the sutures removed prior to the experiment. The wound was ruptured on either side of it. Two determinations were made of the tensile strength of each wound.

Viscose cellulose sponges 10 × 10 × 20 mm (Vibella Säteri Oy, Valkeakoski, Finland) were divided into two equal parts and immediately sewn together with silk suture. The pair implanted in each animal was of about the same weight. The animals were anesthetized with the same and two symmetrical incisions of 2 cm to the midline of the back were made. A s.c. pocket was dissected in each wound. The cellulose sponges had been soaked in serum or wound fluid prior to the implantation in the pockets. Each animal got one untreated sponge on one of the pockets and wound fluid-treated sponge in the other.

TABLE I

	T.S.	Significance	N
control	553 \pm 25	p 0.01 0.02	68
	466 \pm 23		68
wound fluid	454 \pm 21	p > 0.20 0.30	78
	418 \pm 19		78
serum	405 \pm 23		71
	420 \pm 23		68

Tensile strength of wounds exposed to wound fluid (WF), serum or control expressed as g Mean \pm S.E. calculation of significance was made using Student's *t*-test. N = number of observations.

7 days later the sponges were removed, and their surfaces dissected free from surrounding tissue. The tensile strength of the sponge was taken away and the tensile strength between the two halves was measured. The dry weight of the sponge was determined after drying at 70° in a vacuum desiccator over phosphorous pentoxide for 3 days.

The tensile strength of the wound on each side was compared to that of the wound on the contralateral side. It was increased in wound exposed to wound fluid, compared to both serum and control, i.e. no treatment. The difference was most pronounced in the group wound fluid/control (Table I).

The dry weight of the sponges did not show any differences in either groups. The same was true for the tensile strength of the sponges implanted under the conditions tested.

Many factors effect wound-healing in animals, such as the general health, the nutritional status and lack of certain vitamins. Each animal has its own characteristic response to an injury. Considering these facts, the present experiments were performed on animals which served as their own control. The exact mechanism could not be demonstrated in the present study. It was, however, possible to prove that wound fluid *per se* promotes the reparative process.

The reason for the increased tensile strength of skin wounds exposed to wound fluid apparently is a change in the connective tissue formed (Dunphy 1960). Its nature is not known. It may be there is a more extensive proliferation of connective tissue cells and formation of collagen (Sandberg and Lederfeldt 1963). However the dry weight of the sponges did not increase significantly probably reflecting that the testing method was not sensitive enough. The tensile strength of the sponges, furthermore, did not show any difference after treatment with wound fluid or serum compared to no pre-treatment, although the pores in the sponges were filled with cells, fibres and amorphous material.

The type of cell proliferating in repairing wounds is of importance. It is likely that they produce or induce production of a factor increasing the tensile strength. The nature of such a factor is not known. It is likely to be formed locally in the wounded area. Most cells are formed in repairing tissue. It is tempting to assume that histamine and biogenic amines may be of importance to healing wounds (Kahlon and Lederfeldt 1960, Sandberg 1962, Sandberg and Lederfeldt 1963, Wasimski *et al.* 1965).

Growth-promoting factors have been demonstrated in several different systems. Par hepatectomy results in liberation of a factor which is humoral. It may increase the frequency of mitosis even in a normal animal transfused with blood from another one subjected to partial hepatectomy (Christensen and Jacobsen 1950, Bucher, Scott and Arb 1951, Wound and Sussman 1951). If embryonic tissue is transplanted to adult animals, this starts to differentiate in the new host. Another such factor is the nerve growth factor which induces proliferation of sympathetic neurons but not of motor neurons. This latter factor is known to be a polypeptide (Levi Montalcini 1973). Thus the conclusion that wound fluid contains a healing promoting factor of unknown composition, influencing the repair process in connective tissue.

References

- BUCHER, N. L. R., J. F. SCOTT and J. C. ARB, Regeneration of the liver in parabiotic rats. *Cancer* 1951 11 457-465.
- CHRISTENSEN, B. G. and E. JACOBSEN, Studies on liver regeneration. *Acta med. scand.* 1950 Suppl. 103-108.
- DUNPHY, J. E., On the nature and care of wounds. *Ann. roy. Coll. Surg. Engl.* 1960, 26, 89.
- GRINDLAY, J. H. and J. M. WANGH, Plastic sponge which acts as a framework for living tissue. *J. Surg.* 1951 63 288-297.
- HOWES, E. L., J. W. SOOY and S. C. HARVEY, The healing of wounds as determined by their tensile strength. *J. Amer. med. Ass.* 1929 92 42-45.
- HUNT, T. K., P. L. TWOMEY, B. M. LEDERFELD and J. E. DUNPHY, Respiratory gas tensions and pH in healing wound. *Amer. J. Surg.* 1967 114 302-307.
- KARLSON, U. and B. LEDERFELD, Association between histamine forming capacity and reparative power. *Acta chir. scand.* 1960, 119 207-208.
- LEVI-MONTALCINI, R., Control mechanisms of the adrenergic neuron. In: *Dynamics of Degeneration and Growth in Nervous System* (ed. K. Fuxe, L. Olsson and Y. Zotterman). Oxford, Pergamon Press, 1973, 207-211.
- SANDBERG, N., Accelerated collagen formation and histamine. *Nature (Lond.)* 1962, 194 183.
- SANDBERG, N. and B. LEDERFELD, The tensile strength of healing wounds and collagen formation in rats and rabbits. *Acta chir. scand.* 1963 126 187.
- SANDBLOM, PH., P. PETERSEN and A. MUREN, Determinations of the tensile strength of the healing wound as clinical test. *Acta chir. scand.* 1953, 105 25-257.
- SCHILLING, J. A., W. JOEL and H. W. STURLEY, Wound-healing, a comparative study of the histological changes in granulation tissue contained in stainless steel wire mesh and polyethylene sponge cylinders. *Surgery* 1959 46 702.
- WASLINSKI, Cz., J. STERNIK, J. MAGROWICZ, R. BILSKI, W. KASLINSKI, A. PRZYBYLSKI and W. M. KOWIAK, *La Chénostation*. C.N.R.S., Paris, 1965 P. 227.
- WINDICKER, A. S. and H. SUSSMAN, Regeneration of liver tissues following partial hepatectomy in parabiotic rats. *Proc. Soc. exp. Biol. (N.Y.)* 1951 76 683-686.

The predominant molecular form of gastrin and cholecystokinin in the gut is a small peptide corresponding to their COOH-terminal tetrapeptide amide

By

JENS F. REHFELD and LARS-INGE LARSEN

peptide hormones in tissue and blood are analyzed by immunochemical methods. In view of the molecular heterogeneity of each hormone, the structural homology between groups of hormones, and the occurrence of hormonal peptides in nerves, immunochemical measurements require exact knowledge of the sequence for which an antiserum is specific. We have recently developed a panel of sequence-specific antisera for radioimmunoassay (Rehfeld 1978 a) and immunocytochemistry (Larsen and Rehfeld 1977) of the two related peptide hormones, gastrin and cholecystokinin (CCK). Using this panel to monitor fractionations, we noted that antisera specific for the common COOH-terminal tetrapeptide amide sequence detected a new small component in extracts from the gut (Rehfeld 1978 b). Moreover, the COOH-terminal-specific antisera demonstrated more cells than were seen with antisera against NH₂-terminal and middle regions of gastrin₁₇, CCK₈ and CCK₃₃ (Larsen and Rehfeld 1979). The COOH-terminal-reactive cells that contained neither CCK₈, CCK₃₃, nor gastrin₁₇ represents a new type of gastrointestinal endocrine cell (the TG-cell), which has now been identified at the electron microscopical level (Larsen and Rehfeld 1979 and Fig. 1). From atrial extracts we purified the new component and demonstrated its identity with the tetrapeptide amide common to gastrin and CCK (unpublished). In the following, we report the distribution and concentrations of the tetrapeptide amide in the gastrointestinal tract.

Mucosa from the gastrointestinal tract of three pigs was collected at a local abattoir 20 min post mortem. The mucosa was immediately frozen on dry ice and stored at -80°C. Mucosa from the mucosa was cut into small pieces and extracted in boiling water and acetic acid, as described in detail elsewhere (Rehfeld 1978 b). 2-6 ml extract was applied to high-resolution Sephadex columns (G-50 superfine, 2.5 × 2000 mm), eluted and calibrated as described in detail elsewhere (Rehfeld 1978 b). The immunoreactivity in extracts and chromatographic fractions was quantitated by an assay specific for the COOH-terminal sequence of gastrin and CCK using antiserum 2609 (Rehfeld *et al.* 1972, Rehfeld 1978 a) mono-oxidized gastrin₁₇ and highly purified porcine gastrin₁₇, CCK₃₃, and synthetic tetrapeptide amide as standards.

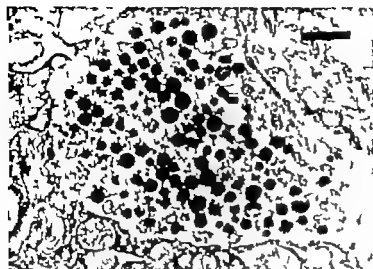


Fig. 1. Electron micrograph of immunocytochemical localization of TG cell processes in the duodenal Brunner gland area of a primate. Bar indicates 1 μ m.

Table I shows that the tetrapeptide-like component on a molar base predominates throughout the gastrointestinal tract in comparison with gastrin₁₇ and CCK₂₈. In antrum gastrin was present in almost half the concentration of that of the tetrapeptide-like component whereas the tetrapeptide predominated even more in distal regions of the gut.

Since the tetrapeptide amide contains essentially all the biological information present in gastrin and CCK and since the tetrapeptide-like component predominates in all regions of the gut, it is tempting to suggest that the new fifth component of gastrin and CCK is its principal molecular form of both hormones. It is possible that the tetrapeptide amide is

TABLE I Regional concentration (pmol/g mucosa (wet weight)) of gastrin₁₇, cholecystokinin and its common CCK terminal tetrapeptide amide in the porcine gastrointestinal tract (mean \pm SD) (n = 3).

	G	G/CCK ₄	CCK ₂₈
Antrum	5 921 \pm 312	13 170 \pm 5 070	<0.1
Duodenum (proximal)	3.1 \pm 1.0	8 220 \pm 3 180	509 \pm 183
Duodenum (middle)	1.2 \pm 0.5	10 740 \pm 3 410	650 \pm 115
Duodenum (distal)	<0.2	3 810 \pm 960	82 \pm 19
Jejunum (proximal)	<0.2	2 790 \pm 1 040	478 \pm 176
Jejunum (middle)	<0.2	1 180 \pm 470	327 \pm 87
Jejunum (distal)	<0.1	108 \pm 51	16.1 \pm 6.0
Ileum (proximal)	<0.2	291 \pm 92	<0.2
Ileum (middle)	<0.2	113 \pm 17	<0.2
Ileum (distal)	<0.2	48 \pm 22	<0.2
Colon (proximal)	<0.2	59 \pm 18	<0.2

its effects locally by paracrine secretion, since it may be cleared from circulation in the liver. In the colon of most species gastrins and CCKs are localized exclusively to neurons (Nilsson-Wallengren *et al* 1977, Larsson and Rehfeld 1978 b). Thus the tetrapeptide is of neuronal origin, as previously demonstrated in the central nervous system (Rehfeld 1978 b, Larsson and Rehfeld 1978 b). Due to its high concentration and small size the tetrapeptide may well turn out to be the principal neurotransmitter of the gastrin-CCK family.

References

- REHFIELD, L.-I. and J. F. REHFIELD, Characterization of antral gastric cells with region-specific antisera. *J. Histochem. Cytochem.* 1977 25 1317-1321.
- REHFIELD, L.-I. and J. F. REHFIELD, Localization and molecular heterogeneity of cholecystokinin in the central and peripheral nervous systems. *Brain Res.* 1978 b, 159 in press.
- REHFIELD, L.-I. and J. F. REHFIELD, Production of a peptide resembling the C-terminus of gastrin by a new gastrointestinal endocrine cell type. 1979 Submitted.
- REHFIELD, J. F. Immunohistochemical studies on cholecystokinin. I. Development of sequence-specific radioimmunoassays for porcine trypsinogen-cholecystokinin. *J. Biol. Chem.* 1978 a, 253, 4016-4021.
- REHFIELD, J. F. Immunohistochemical studies on cholecystokinin. II. Distribution and molecular heterogeneity in the central nervous system and small intestine of man and hog. *J. Biol. Chem.* 1978 b, 253, 4022-4030.
- REHFIELD, J. F., F. S. ADRI and B. RUTEN, Production and evaluation of antibodies for the radioimmunoassay of gastrin. *Scand. J. Clin. Lab. Invest.* 1972 30, 221-232.
- NILSSON-WALLENNGREN, K., J. F. REHFIELD, L.-I. LARSSON and B. UYVÄL, Hapendecapeptide gastrin in the rat. *Proc. nat. Acad. Sci. (Wash.)* 1977 74 5707-5710.

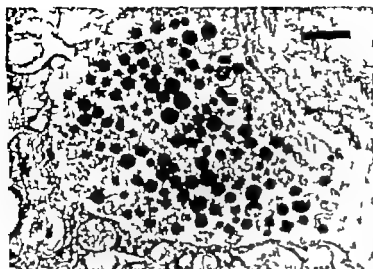


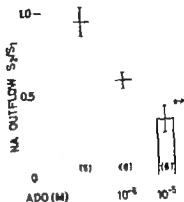
Fig. 1 Electron micrograph of immunocytochemical test-TG cell process in the distal Brunner gland area of a monkey. Bar indicates 1 μ m.

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TABLE I. Regional concentration (pmol/g mucosa (wet weight)) of gastrin₁₇, cholecystokinin₂₈ and the common COOH-terminal tetrapeptide amide in the porcine gastrointestinal tract (mean \pm S.E. (n = 3)).

	G	G/CCK	CCK ₂₈
Antrum	59.1 \pm 2.31	13.170 \pm 3.070	<0.2
Duodenum (proximal)	3.1 \pm 1.0	8.220 \pm 3.180	509 \pm 183
Duodenum (middle)	1.2 \pm 0.5	10.740 \pm 3.410	630 \pm 113
Duodenum (distal)	<0.2	3.810 \pm 960	82 \pm 19
Jejunum (proximal)	<0.2	2.790 \pm 1.040	478 \pm 176
Jejunum (middle)	<0.2	1.180 \pm 420	327 \pm 87
Jejunum (distal)	<0.2	108 \pm 51	16.1 \pm 6.0
Ileum (proximal)	<0.2	291 \pm 92	<0.2
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1. Effect of adenosine (ADO) on NA release in response to sympathetic nerve stimulation (10 Hz, 30 s) in perfused hearts. Values presented as ratio between two consecutive measurements and given as means \pm S.E. Figures in parentheses number of experiments.

range adenosine markedly and dose-dependently depressed the release of NA elicited by adrenergic nerve stimulation (Fig. 1). The mechanical responses were also depressed. Adenosine effects were rapidly reversed when the infusion was discontinued. Adenosine $1 \mu\text{M}$ inhibited the chronotropic and inotropic responses to nerve stimulation 19.7 ± 3.4 and 39.2 ± 4.0 (means \pm S.E., $n = 7$) percent, respectively. At this concentration adenosine had no effect on the contractile force and only inconsistently and slightly (less than 5%) depressed the heart rate. Seemingly adenosine $10 \mu\text{M}$ more effectively depressed the mechanical responses to nerve stimulation. However quantitative analysis of these data is not appear meaningful since $10 \mu\text{M}$ adenosine *per se* markedly depressed heart rate and contractile force.

Ample experimental evidence suggests that adenosine is a physiological regulator of coronary blood flow during hypoxia or increased oxygen demand (Berne 1963, Rubio and me 1969). The present finding that adenosine inhibits NA overflow and effector responses to adrenergic nerve stimulation may represent another significant level of adenosine action on the heart. It is very unlikely that the inhibition of NA overflow was secondary to increased perfusion flow since maximal vasodilatation occurred already with $1 \mu\text{M}$ adenosine. Moreover vasodilatation is expected to cause, if anything, increased washout of liberated transmitter. It may be concluded, therefore, that adenosine inhibits adrenergic transmission in the heart by a prejunctional action on NA release, as has been demonstrated in several other tissues from different animal species (Hedqvist and Fredholm 1976, Envero and Saidman 1977, Verhaeghe *et al.* 1977).

Recently adenosine was shown to inhibit chronotropic and inotropic responses to catecholamines in the guinea pig heart (Schneider *et al.* 1977). There is no compelling reason to assume that this effect is confined to the guinea pig heart or that inhibition of NA release occurs only in the rabbit heart. Hence it follows that adenosine-mediated modulation of adrenergic transmission in cardiac tissue may involve both a prejunctional and postjunctional component that are complementary to each other.

There is considerable evidence to show that adrenergic nerve stimulation causes an increased release of adenosine in a number of tissues, such as canine adipose tissue, rabbit duodenum and heart and guinea pig vas deferens (Fredholm and Hedqvist 1978). In the rabbit

Inhibitory effect of adenosine on adrenergic neuroeffector transmission in the rabbit heart

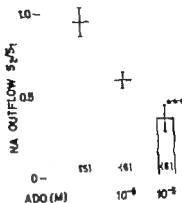
By

PER HEDQVIST and BERTIL B. FREDHOLM

Adenosine is a potent coronary vasodilator and has been implicated as a mediator of cardiac blood flow regulation (Wedd 1931 Berne 1963 Rubio and Berne 1969). Furthermore, adenosine decreases heart rate (Wedd 1931) and antagonizes chronotropic and inotropic responses to catecholamines (Schrader *et al* 1977). Recent observations indicate that adenosine may inhibit noradrenaline (NA) release in a number of tissues, such as later adipose tissue, vas deferens and vascular strips (Hedqvist and Fredholm 1976, Enerbom & Saidman 1977 Verhaeghe *et al* 1977). The present study indicates that such a mechanism is operative also in the heart.

Rabbits of either sex weighing 1.5-2.0 kg, received heparin 1 000 IU/kg b.wt. iv and were killed by a blow on the head. After exsanguination the heart with its sympathetic nervous supply was dissected free, as described by Huković and Muscholl (1962) and perfused according to the Langendorff technique with Tyrode's solution, at a constant pressure of 75 cm H₂O of the following composition (conc. in mM): NaCl 136.7, KCl 4.0, CaCl₂ 1.8, MgCl₂ 1.0, NaHCO₃ 11.9, NaH₂PO₄ 0.4, glucose 5.5, ascorbic acid 0.1 and at 37°C and gassed with 5% CO₂ in O₂. The sympathetic nerves were pulled through platinum ring electrodes and were stimulated with two Grass S4 stimulators, delivering 30 s train biphasic pulses (10 Hz, 1 ms, 20 V effective strength) at intervals of 10 min. The myocardial contractile force and heart rate were monitored by means of a force displacement transducer (Grass FT 03) connected to a hook in the ventricle, or a pressure transducer (Statham 1Dc) connected to a small rubber balloon in the left ventricle. Either parameter was displayed on a Grass polygraph. Perfusate from the heart was collected for two min immediately preceding a stimulation, and during and 90 s after the stimulation. The NA content in perfusate samples was determined fluorimetrically according to Euler and Lishajko (1969). Adenosine was infused into the perfusion stream close to the heart to give a final concentration of 1 or 10 μ M.

In the Langendorff-perfused rabbit heart adenosine (1 and 10 μ M) significantly increased coronary drainage in agreement with previous reports that adenosine is a vasodilator in mammalian heart (Wedd 1931 Berne 1963). The two concentrations caused 29.1 ± 6.1 and 33.2 ± 4.1 (means \pm S.E., $n=8$) percent increase in flow rate, respectively, indicating that maximal vasodilatation was achieved already with 1 μ M adenosine. In the same concentration



1 Effect of adenosine (ADO) on NA release in response to sympathetic nerve stimulation (10 Hz, 30 s) in perfused rat hearts. Values presented as ratio between two consecutive stimulations and given as means \pm S.E. Figures in brackets number of experiments.

Adenosine markedly and dose-dependently depressed the release of NA elicited by sympathetic nerve stimulation (Fig. 1). The mechanical responses were also depressed. Adenosine effects were rapidly reversed when the infusion was discontinued. Adenosine 10^{-5} M inhibited the chronotropic and inotropic responses to nerve stimulation 19.7 ± 3.4 and 39.2 ± 4.0 (means \pm S.E., $n = 7$) percent, respectively. At this concentration adenosine had no effect on the contractile force and only inconsistently and slightly (less than 5%) depressed the heart rate. Seemingly adenosine 10^{-4} M more effectively depressed the mechanical responses to nerve stimulation. However, quantitative analysis of these data does not appear meaningful since 10^{-4} M adenosine *per se* markedly depressed heart rate and contractile force.

Ample experimental evidence suggests that adenosine is a physiological regulator of coronary blood flow during hypoxia or increased oxygen demand (Berne 1963, Rubio and Sme 1969). The present finding that adenosine inhibits NA overflow and effector responses to sympathetic nerve stimulation may represent another significant level of adenosine action on the heart. It is very unlikely that the inhibition of NA overflow was secondary to increased coronary flow since maximal vasodilatation occurred already with 10^{-5} M adenosine. More vasodilatation is expected to cause, if anything, increased washout of liberated transmitter. It may be concluded, therefore, that adenosine inhibits adrenergic transmission in the heart by a prejunctional action on NA release, as has been demonstrated in several other tissues from different animal species (Hedqvist and Fredholm 1976, Entro and Saldana 1977, Verhaeghe *et al.* 1977).

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heart basal level of adenosine was very low (less than $0.05 \mu\text{M}$) but increased to approximately $0.3 \mu\text{M}$ in response to adrenergic nerve stimulation (10 Hz, 30 s). This value is far from the adenosine concentration ($1 \mu\text{M}$) which in the present study was found to significantly depress the release of NA. Moreover rapid inactivation of adenosine, reflected by less than 1/3 of infused adenosine escaping passage through the coronary circulation (Fredholm and Hedqvist 1978) would seem to minimize or even mask the difference. It appears very likely therefore, that in the Langendorff-perfused rabbit heart nerve activity releases adenosine in concentrations that suffice to exert a negative-feedback control of adrenergic transmission. Since adenosine is also released by the normal myocardium (Rubio and Berne 1969), a physiological role for adenosine on adrenergic transmission must be considered.

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References

- BERNE, R. M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *J. Physiol.* 1963, 104, 317-322.
- ENDO, M. A. and B. Q. SAIDMAN. Possible feed-back inhibition of noradrenaline release by post-synaptic compounds. *Neuro-Schmidberg Arch. Pharmacol.* 1977, 297, 39-46.
- VON EULER, U. S. and F. LIDHARTO. Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. scand.* 1961, 51, 348-355.
- FREDHOLM, B. B. and P. HEDQVIST. Adenosine—a transsynaptic modulator of noradrenergic release. *Catecholamines: Basic and clinical frontiers*. Ed. E. Usdin. Pergamon Press, Oxford & New York, 1978, in press.
- HEDQVIST, P. and B. B. FREDHOLM. Effects of adenosine on adrenergic neurotransmission. Presynaptic inhibition and postjunctional enhancement. *Neuro-Schmidberg Arch. Pharmacol.* 1976, 295, 217-224.
- HUKOVIĆ, S. and E. MUSCHOLL. Die Noradrenalin-Abgabe aus dem isolierten Kaninchenherzen bei sympathischer Nervenreizung und ihre pharmakologische Beeinflussung. *Neuro-Schmidberg Arch. Pharmacol.* 1962, 244, 81-96.
- RUBIO, R. and R. M. BERNE. Release of adenosine by the normal myocardium in dogs and its relation to the regulation of coronary resistance. *Circulat. Res.* 1969, 25, 407-415.
- SCHRAEDER, J. G. BAUMANN and E. GERLACH. Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Arch.* 1977, 372, 29-35.
- VERHAEGHE, R. H. P. M. VANHOUTTE and J. T. SHEPARD. Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenosine nucleotides. *Circulat. Res.* 1977, 40, 208-215.
- WIDD, A. M. The action of adenosine and certain related compounds on the coronary flow of the perfused heart of the rabbit. *J. Pharmacol. exp. Ther.* 1931, 41, 355-366.

Inhibition of thirst and apparent ADH release by intracerebroventricular ethacrynic acid

By

M. RUNDGREN, M. J. MCKINLEY, L. G. LIDSELL and B. ANDERSSON

ies performed in the goat over the past several years imply that cerebral receptors sensing water intake and the release of antidiuretic hormone (ADH) have a juxtaventricular location, and that the activity of these receptors is positively correlated to the $[Na^+]$ in the cerebrospinal fluid (CSF). That Na^+ -transporting enzyme activity in some way may be essential for the excitation of the receptors is indicated by the observations that intracerebroventricular (IVT) infusions of certain stimulants of active Na^+ -transport interact with CSF Na^+ in eliciting thirst and ADH-release (cf. Andersson 1978). This possibility is also supported by effects of Na^+ -K-ATPase inhibitors so far reported in three species. In the rat hypothalamic implants of ouabain suppressed water intake (Bergmann *et al.* 1967) and induced a water diuresis (Gutman, Bergmann and Zerachia 1971). In the sheep the carotid infusion of ouabain virtually abolished water intake in response to intracarotid infusions of hypertonic NaCl and angiotensin II and decreased the urge to drink in water-deprived animals (Weisberger, Denton and McKinley 1977). In the goat, IVT infusions of two weaker inhibitors of Na^+ -K-ATPase (glycerol and deuterium) prevented drinking in water-deprived animals and attenuated the basic ADH secretion of goats in normal water intake (Olsson, Larsson and Liljekvist 1976, Lidsell, Liljakko and Rundgren 1976). Therefore, it was of interest to study whether another inhibitor of enzymatic Na^+ -transport, ethacrynic acid (cf. Schwartz, Lindenmayer and Allen 1975), would affect the cerebral control of water balance in a similar manner.

Two female, and two castrated male goats (b.wt. 31-36 kg) were used. The animals were implanted with permanent platinum-iridium cannulas (Åkerlund, Andersson and Olsson 1973) in the lateral ventricle where IVT infusions were made at a rate of 20 μ l/min. The sodium salt of ethacrynic acid (Edecrin, Merck S & D[®]) was used for IVT infusions.

Thirst. In the castrated male goats IVT infusions of ethacrynic acid (0.3 μ g/kg min^{-1}) dissolved in 0.3 M NaCl, or merely 0.3 M NaCl were alternately made for 60 min. During the first 55 min the water bucket in front of the goats was covered with a lid. This lid was temporarily removed at 10 min intervals to test the willingness of the goats to drink. They

Holder of an Australian National Health and Medical Research Council C. J. Martin Fellowship. On leave from the Howard Flacey Institute of Experimental Physiology and Medicine, University of Melbourne.

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- BERNE, R. M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *Am. J. Physiol.* 1963 204 317-322.
- ENERG, M. A. and B. Q. SAIDMAN. Possible feed-back inhibition of norepinephrine release by paracrine compounds. *Naunyn-Schmiedeberg Arch. Pharmacol.* 1977 297 39-46.
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References

- BERNE, R. M. Cardiac nucleotides in hypoxia: possible role in regulation of coronary blood flow. *J. Physiol.* 1963 204 317-322.
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- HEDQVIST, P. and B. B. FREDHOLM, Effects of adenosine on adrenergic neurotransmission. Prejunctional inhibition and postjunctional enhancement. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1976 291 217-221.
- HUKOVIC, S. and E. MUSCHOLL, Die Noradrenalin-Abgabe aus dem isolierten Kaninchenherzen bei parasympathischer Nervenreizung und ihre pharmakologische Beeinflussung. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1966 244 81-96.
- RUBIO, R. and R. M. BERNE, Release of adenosine by the normal myocardium in dogs and its relationship to the regulation of coronary resistance. *Circulat. Res.* 1969 25 407-415.
- SCHRADER, J. H., B. BAUMANN and E. GERLACH, Adenosine as inhibitor of myocardial effects of catecholamines. *Pflügers Arch.* 1977 372 29-35.
- VERHAEGE, R. H., P. M. VANHOUTTE and J. T. SHEPARD, Inhibition of sympathetic neurotransmission in canine blood vessels by adenosine and adenine nucleotides. *Circulat. Res.* 1977 40 208-215.
- WEDD, A. M. The action of adenosine and certain related compounds on the coronary flow of the perfused heart of the rabbit. *J. Pharmacol. exp. Ther.* 1931 41 355-366.

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References

- ANDERSON, L.-E., I. ANDERSSON and K. OLSSON, A cannula system for frequent infusions into the CSF of the cerebral ventricles of the goat. *Physiol. Behav.* 1973 10, 161-162.
- ANDERSSON, I. Thirst—end brain control of water balance. *Amer. Sci.* 1971 59, 408-415.
- ANDERSSON, I. Regulation of water intake. *Physiol. Rev.* 1978 58, 583-603.
- CHALOVITZ, F., M. CHALOVITZ, A. COHEN, Y. GUTMAN and Y. GEMAT, Water intake of rats after micro-injections of osmotic into the hypothalamus. *Amer. J. Physiol.* 1967 215, 325-332.
- CHALOVITZ, Y., F. CHALOVITZ and A. ZERACHIA, Influence of hypothalamic deposits of antidiuretic drugs on water excretion. *Europ. J. Pharmacol.* 1971 13, 325-329.
- CELL, L. G., F. LARSSON and M. RINGDÖREN, Negative water balance induced by intracerebroventricular infusions of desmopressin. *Acta physiol. scand.* 1976 97, 142-144.
- CELL, L. G., F. LARSSON and E. LILJEVIST, Intracerebroventricular glycerol: A potent inhibitor of ADH release and thirst. *Acta physiol. scand.* 1976 98, 470-477.
- FRANZ, A., G. E. LINDENMAYER and J. C. ALLAN, The sodium-potassium adenosine triphosphatase. Pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.* 1975, 27, 3-134.
- JONES, T. S., L. SKOLD, L. BONTING and W. W. OFFELL, Na-K-activated adenosine triphosphatase formation of cerebrospinal fluid in the cat. *Amer. J. Physiol.* 1966 206, 1165-1172.
- KERLEY, R. S., D. A. DENTON and M. J. MCKINLEY, Inhibition of water intake by osmotic administration to sheep. *Pharmacol. Biochem. Behav.* 1977 7, 121-128.

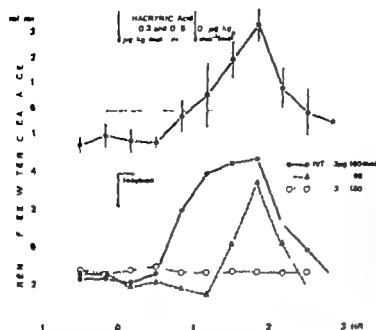


Fig. 1 Above: Mean effect on free water clearance of ethacrynic acid infusions into the lateral ventricle ($n=4$) of two somewhat dehydrated goats. Vertical bars: S.E. Below: The response to IVT ethacrynic acid at 0.3 and 0.1 $\mu\text{g}/\text{kg min}^{-1}$ in the animals. For comparison shown the absence of effects in response to an intravenous infusion of the larger dose.

were not allowed to consume any water however. After 55 min the goats got free access to water. Both kinds of infusion induced water seeking behaviour within 10 min. However, during the ethacrynic acid/NaCl infusions the urge to drink disappeared 20 to 40 min later. Hence, no water was drunk when the animals got free access to it. In contrast, when 0.3 M NaCl was infused the goats drank considerable amounts of water at this stage (table below). The ethacrynic acid infusions did not affect the appetite of the goats or any other visible signs of discomfort.

Lateral ventricle (20 $\mu\text{l}/\text{min}$ 60 min)	Water intake at 55 min (ml, Mean \pm S.E.)	Exp. no.
0.3 M NaCl	860 \pm 170	6
Ethacrynic acid in 0.3 M NaCl	0	6

ADH secretion In the two female goats ethacrynic acid was infused into the lateral ventricle dissolved in slightly hypertonic (0.17 M) NaCl. In one of the goats the doses were 0.3 and 0.15 $\mu\text{g}/\text{kg min}^{-1}$ (duration 60 min) and in the other animal 0.3 and 0.1 $\mu\text{g}/\text{kg min}^{-1}$ (duration 60 and 90 min respectively). All infusions induced positive renal free water clearance (C_{H_2O}) towards the end of the infusion period. However, at the lowest acid concentration C_{H_2O} did not become positive until after about 80 min of infusion. Intravenous control infusions (0.3 $\mu\text{g}/\text{kg min}^{-1}$ for 60 min) did not induce any water excretion. The mean effect on renal C_{H_2O} and the individual results in one of the goats are shown in Fig. 1. Neither the IVT nor the I.V. infusions had any obvious effect upon renal Na⁺ and K⁺ excretion.

Discussion This study shows that the administration of systemically inactive amounts of ethacrynic acid into the CSF of the lateral ventricle can block the diuretic effect of

infusions of hypertonic NaCl and induce a water diuresis in the nonhydrated animal without having other obvious effects. It provides additional evidence for the concept that osmotic receptors involved in the regulation of water intake and ADH secretion are in contact with the CSF and are susceptible to changes in its composition (Andersson 1971). Since ethacrynic acid inhibits Na-K-ATPase (cf Schwartz *et al.* 1975), the experiments also lend support to the idea that active enzymatic cation transport forms an essential part of the receptor excitation process. However, a more indirect action of ethacrynic acid at the receptor level cannot be excluded as explanation for the inhibitory effects observed in the present study. Choroidal Na-K ATPase activity is a prerequisite for Na transport from the blood to the CSF (Vaites *et al.* 1974), and IVT infusions of a weak inhibitor of Na-K-ATPase (glycerol) have recently been observed to lower the CSF [Na] more effectively than corresponding glucose infusions (Olsson *et al.* 1976). In the IVT infusions of ethacrynic acid might have lowered the [Na] of the CSF and in that manner indirectly have reduced the activity of juxta-ventricular sodium sensitive receptors involved in the control of water balance. Therefore, it would be of interest to extend this rather preliminary study to involve also pre- and post-infusion determinations of [Na].

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References

- ANDERSSON, L. E. I. ANDERSSON and K. OLSSON, A cannula system for frequent infusions into the CSF of the cerebral ventricles of the goat. *Physiol. Behav.* 1973, 10, 161-162.
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- ANDERSSON, B. Regulation of water intake. *Physiol. Rev.* 1970, 50, 582-603.
- BERGMANN, F. M. CHADKOVITZ, A. COSTA, Y. GUTHRIE and Y. GRATT, Water intake of rats after implantation of cannulae into the hypothalamus. *Amer. J. Physiol.* 1967, 213, 328-332.
- FRAN, Y. F. BERGMANN and A. ZERACHIA, Influence of hypothalamic deposits of osmolytic drugs on water excretion. *Europ. J. Pharmacol.* 1971, 15, 326-329.
- GALL, L. O. F. LINDAHL and M. RUNDQVIST, Negative water balance induced by intracerebroventricular release of deoxycorticosterone. *Acta physiol. scand.* 1976, 97, 143-144.
- MOY, K., B. LARSEN and E. LARSEN, Intracerebroventricular glycerol: A potent inhibitor of ADH release and thirst. *Acta physiol. scand.* 1976, 98, 470-477.
- WERNITZ, A., G. E. LINDENMAYER and J. C. ALLEN, The sodium-potassium adenosine triphosphatase: Pharmacological, physiological and biochemical aspects. *Pharmacol. Rev.* 1975, 27, 3-134.
- JOHNSON, T. S., L. S. JOHNSON, L. BONTING and W. W. OPPEN, Na-K-activated adenosine triphosphatase formation of cerebrospinal fluid in the cat. *Amer. J. Physiol.* 1964, 206, 1165-1172.
- FRANZBLAU, R. S., D. A. DENTON and M. J. MCKINLEY, Inhibition of water intake by osmotic administration in sheep. *Pharmacol. Biochem. Behav.* 1977, 7, 121-128.

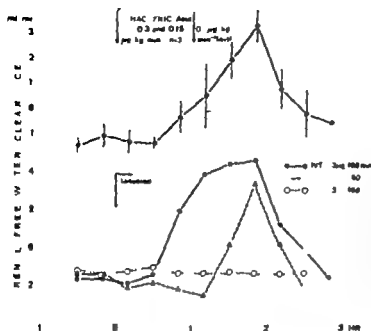


Fig. 1 Above: Mean effect-free water clearance of ethacrynic acid infusions into the lateral ventricle ($n=4$) of two normally non-hydrated goats. Verticals: S.E. Below: The same applies to IVT ethacrynic at 0.3 and 0.1 $\mu\text{g}/\text{kg min}$ as the animals. For comparison shown the absence of effect as to response to an isosmotic infusion of the larger dose.

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Lateral ventricle (20 $\mu\text{l}/\text{min}$ 60 min)	Water intake at 55 min (ml Mean \pm S.E.)	Exp. no.
0.3 M NaCl	860 \pm 170	8
Ethacrynic acid in 0.3 M NaCl	0	6

ADH secretion In the two female goats ethacrynic acid was infused into the lateral ventricle dissolved in slightly hypertonic (0.17 M) NaCl. In one of the goats the doses were 0.3 and 0.15 $\mu\text{g}/\text{kg min}$ (duration 60 min) and in the other animal 0.3 and 0.1 $\mu\text{g}/\text{kg min}$ (duration 60 and 90 min respectively). All infusions induced positive renal free water clearance (C_{H_2O}) towards the end of the infusion period. However, at the lowest ethacrynic acid concentration C_{H_2O} did not become positive until after about 80 min of infusion. Intravenous control infusions (0.3 $\mu\text{g}/\text{kg min}$ for 60 min) did not induce any water diuresis. The mean effect on renal C_{H_2O} and the individual results in one of the goats are shown in Fig. 1. Neither the IVT nor the i.v. infusions had any obvious effect upon renal Na⁺ and K⁺ excretion.

Discussion. This study shows that the administration of systemically inactive amounts of ethacrynic acid into the CSF of the lateral ventricle can block the dipsogenic response

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References

1. JELLS, L.-E. 1. ANDERSSON and K. OLSSON, A cannula system for frequent infusions into the CSF of the cerebral ventricles of the goat. *Physiol. Behav.* 1973, 10, 161-162.
2. ANDERSSON, K. Thirst and brain control of water balance. *Ann. Sci.* 1971, 28, 405-415.
3. ANDERSSON, K. Regulation of water intake. *Physiol. Rev.* 1972, 52, 582-601.
4. OLSSON, K., M. CHADMOVITZ, A. COETM, Y. GUTMAN and Y. ORNAT, Water intake of rats after injections of ouabain into the hypothalamus. *Amer. J. Physiol.* 1967, 215, 328-332.
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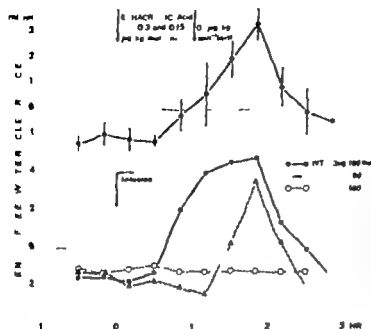


Fig. 1. Effect of free water clearance of ethacrynic acid infusions into the lateral ventricle ($n=4$) of two normal nonhydrated goats. Vertical S.E. Arrows. The square responses to IVT ethacrynic acid 0.3 and 0.1 $\mu\text{g}/\text{kg}\cdot\text{min}$ in the animals. For comparison shown the absence of similar response to an intravenous infusion of the larger dose.

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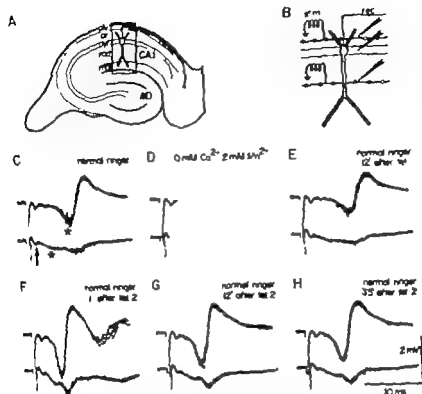


Fig. 1. A. Schematic drawing of the hippocampal slice. B. In the circled region of CA1 framed, str. alveus, stratum oriens, pyr. stratum pyramidale-rad., stratum radiatum, mol., stratum moleculare. C. Enlarged view of area shown in A, showing positioning of stimulating (stim) and recording (rec) electrodes. D. Results from an experiment showing the field potentials evoked by stimulation of str. radiatum (stimulated pathway). Each pair of traces consists of 5 superimposed recordings of potentials from str. pyramidale (upper trace) and str. radiatum (lower trace). C. Control, showing presynaptic volley (v), field potential (f), and population spikes (sp). D. Synaptic transmission blocked by 0 mM Ca^{2+} 2 mM Mn^{2+} recorded immediately before tetanus 1 (50 Hz at 5 s). E. Recovery of synaptic potentials 1 min after tetanus 1 showing absence of LTP recorded immediately before tetanus (50 Hz at 5 s). F-H. Potentiation of field potential and population spikes, recorded 1 min (F), 12 min (G), and 35 min (H) after tetanus 2. Length of oscilloscope traces are photographed for only 35 min after the second tetanus on-line measurements showed that LTP persisted virtually unchanged for as long as the experiment was continued (duration 1 LTP 2 h).

After switching back to the normal fluid, but while synaptic transmission was still blocked, a tetanus was given (arrow tet 1). Within 15 min after the tetanus the responses of both sides recovered to their initial values, showing that no LTP had been produced. A second tetanus in normal solution (arrow tet 2) resulted in potentiation which outlasted the duration of the experiment (87 min). Results consistent with those described above were obtained in all of 5 expts. using calcium-free Ringer and in 2 additional expts. using 1 mM Ca^{2+} 4 mM Mn^{2+} to block synaptic transmission.

Measurements of the presynaptic volley during tetani showed that the amplitudes of the volley during tetanic and blocked synaptic transmission were essentially the same. This

Calcium dependency of synaptic long-lasting potentiation in the hippocampal slice

By

H WIGSTRÖM, J W SWANN and P ANDERSEN

Long lasting potentiation (LLP) of synaptic responses following short trains (tetanic electric stimulation) is an outstanding feature of the hippocampal formation (cf. Lomo 1973, Schwartzkroin and Wester 1975). Little is still known about the mechanism underlying LLP though increased transmitter output has been considered a likely possibility (Andersen *et al.* 1977). The results presented here demonstrate that the presence of extracellular calcium during the tetanus is necessary for the development of LLP.

Experiments were performed on transverse hippocampal slices of guinea pigs (Skrede and Korf 1971). To reduce diffusion times a somewhat modified technique was developed in which embedded slices were used. The experimental situation is described in Fig. 1 A and B. Two synapses to the CA1 pyramidal cells (stratum radiatum and stratum oriens) were stimulated alternately. Potentials were recorded at the levels of the stimulating electrodes and in the cell body layer (cf. Fig. 1). Presynaptic volley (†), field epp (✱), and population spike (✱✱) were measured.

The experiment began with a 15-30 min control period in normal perfusion solution (containing 2 mM Ca^{2+}). Thereafter a solution with a modified composition was delivered producing a blocked synaptic transmission. The latter solution was usually calcium-free (in experiments combined with addition of Mg^{2+} or Mn^{2+}) but solutions with normal 2 mM calcium concentration containing 4 mM aspartate (calcium blocker) were also used. Once synaptic transmission was blocked, tetanus (typically 50 Hz, 5 s) was given to one of the two synaptic inputs. Immediately thereafter or shortly before the tetanus the perfusion solution was changed back to the normal. After synaptic transmission had recovered the presence of long lasting potentiation could be determined by comparing the amplitudes of field epp and population spike of the tetanized side with the previous control values. Since LLP is an input-specific phenomenon (Andersen *et al.* 1977) the responses evoked by stimulation of the non-tetanized side can be used to verify that the preparation fully recovered from the changes in perfusion solution. In order to show that the slices were indeed able to generate LLP a second tetanus was given (in normal solution) and responses were monitored for 1 h thereafter.

Results from one experiment are given in Fig. 1 C-H showing field potential records for the tetanized pathway. The first tetanus given in a state of blocked synaptic transmission (0 mM Ca^{2+} , 2 mM Mn^{2+}) produced no long-lasting potentiation (compare Fig. 2 C and D) while a subsequent tetanus in normal solution did (compare Fig. 2 E and G). The potentiation produced by the second tetanus remained virtually unchanged even after 2 h. Computer generated graphs in Fig. 2 show the time course of another experiment. Field epp, synaptic volley and population spike in response to oriens (tetanized pathway) and radiatum (control) stimulation are plotted. A calcium-free solution was used to block synaptic

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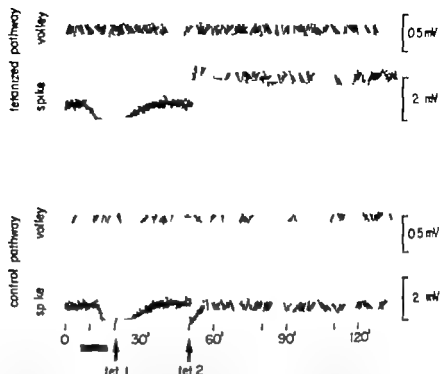


Fig. 2. Presynaptic volley and population spike I response to stimulation of str. oriens (tetanized path, upper plots) and of str. radiatum (control pathway, lower plots). Bar indicates time for perfusion with 0 mM Ca^{2+} solution. Arrows tet 1 and tet 2 mark the two tetani (50 Hz in 5 s, delivered to str. oriens). The stimulation strengths were finely adjusted by a computer in order to maintain constant presynaptic volley amplitudes.

ascertains that our results are not merely due to a failure of the volley to follow the tetanizing frequency during the synaptic blockade.

The results presented here show that the presence of calcium extracellularly—and as likely presynaptic calcium influx—is necessary during the tetanus in order to produce long-lasting potentiation (LLP). It may well be an indirect dependence, however, the mechanism being related to some link in the chain which starts with calcium entry and ends with the postsynaptic potential.

After this manuscript was prepared Dunwiddie *et al.* (1978) published a report on a similar investigation. Our work in part confirms theirs but extends it since we measured not only population spike but also field e.p.s.p. and presynaptic volley.

References

- ANDERSEN, P., S. H. SUNDBERG, O. SVENN and H. WIGSTRÖM, Specific long-lasting potentiation of synaptic transmission in hippocampal slices. *Nature* (Lond.) 1977, 266, 736–737.
- BLISS, T. V. P. and T. LOWE, Long-lasting potentiation of synaptic transmission in the dentate area of an anesthetized rabbit following stimulation of the perforant path. *J. Physiol.* (Lond.) 1973, 232, 331–356.
- DUNWIDDIE, T., D. MASON and G. LYNCH, Synaptic transmission is required for initiation of long-lasting potentiation. *Brain Res.* 1978, 150, 413–417.
- SCHWARTZKROB, P. A. and K. WESTER, Long-lasting facilitation of synaptic potential following tetanization in the in vitro hippocampal slice. *Brain Res.* 1975, 89, 107–119.
- SKERFVING, K. K. and R. H. WESTGAARD, The transverse hippocampal slice: a well-defined cortical maintained in vitro. *Brain Res.* 1971, 35, 589–593.

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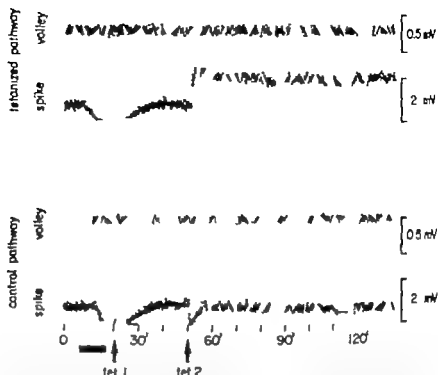


Fig 2. Presynaptic volley and population spike in response to stimulation of str. oriens (retained path; upper plots) and of str. radiatum (control pathway; lower plots). Bar indicates time for perfusion of mM Ca^{2+} solution. Arrows tet 1 and tet 2 mark the two tetani (30 Hz in 5 s, delivered to str. oriens). The stimulation strengths were finely adjusted by a computer in order to maintain constant presynaptic volley amplitudes.

ascertains that our results are not merely due to a failure of the volley to follow the tetanizing frequency during the synaptic blockade.

The results presented here show that the presence of calcium extracellularly—and, likely, presynaptic calcium influx—is necessary during the tetanus in order to produce long-lasting potentiation (LLP). It may well be an indirect dependence, however, the mechanism being related to some link in the chain which starts with calcium entry and ends with the postsynaptic potential.

After this manuscript was prepared Dunwiddie *et al.* (1978) published a report on a similar investigation. Our work in part confirms theirs but extends it since we measure not only population spike but also field epp and presynaptic volley.

References

- ANDERSEN, P., S. H. SUNDBERG, D. SVEN and H. WIGSTRÖM, Specific long-lasting potentiation of synaptic transmission in hippocampal slices. *Nature* (Lond.) 1977, 266, 736–737.
- BILAU, T. V. F. and T. LOWE, Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *J. Physiol.* (Lond.) 1973, 232, 331–356.
- DUNWIDDIE, T., D. MADSON and G. LYNN, Synaptic transmission is required for initiation of long-term potentiation. *Brain Res.* 1978, 150, 413–417.
- SCHWARTZKROB, M. A. and K. WESTER, Long-lasting facilitation of a synaptic potential following tetanization in the in vitro hippocampal slice. *Brain Res.* 1975, 89, 107–119.
- SKERFVING, K. K. and R. H. WESTGAARD, The transverse hippocampal slice: a well-defined cortical structure maintained in vitro. *Brain Res.* 1971, 35, 589–593.

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A phenomenologic evaluation of CO₂-diffusion restriction in kidney tubules studied in an artificial membrane system

By

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Abstract

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chemical events in multi-membrane system with interacting H⁺ and HCO₃⁻ ions has been described phenomenologically as an analogy of the neutralization reaction between secreted H⁺ and filtered HCO₃⁻ in the proximal tubules of the kidney. It is shown that the produced CO₂ gave the highest P_{CO₂} in asymmetrically placed reaction centre, which favours build-up of high intratubular P_{CO₂}. The CO₂ which is dependent on the rate-limiting permeation of the reaction ions, and the permeation could be altered by the influence of solutions of macromolecules such as carbonic anhydrase, albumin and dextran.

Hydrogen ions are formed in the proximal tubular cells of the mammalian kidney and transported into the tubular lumen. Somewhere, the hydrogen ions react with the filtered carbonate ions, but the exact location of this neutralization reaction is unknown. It probably occurs in the neighbourhood of the luminal cell membrane. Carbon dioxide is formed and diffuses to the surroundings by its tension gradients.

The aim of this investigation was to describe phenomenologically the carbon dioxide diffusion out from the tubular lumen and how a build-up of a carbon dioxide difference between the tubular lumen and the tubular cell might occur. If hydrogen ions and bicarbonate ions are permitted to diffuse towards each other, a reaction will take place as follows.



Carbonic acid will be formed, but as k_1 is about 330 times greater than k_2 (Garg *et al.* 1972) an equilibrium will be displaced to the right and carbon dioxide will be produced to a greater extent than carbonic acid. From that part of a system where the ions react with each other carbon dioxide will be formed, and carbon dioxide tension gradients will be built up. Rector *et al.* (1965) and Vieira and Malnic (1968) compared the *in vivo* pH of the proximal tubular fluid with that obtained for the same fluid *in vitro* after equilibration to P_{CO₂} of

40 mmHg. During control conditions this difference was small. After carbonic anhydrase inhibition, however, these mean differences ranged between 0.41 to 0.85 pH units. These were interpreted as a chemical nonequilibrium of the bicarbonate buffer system, induced by the enzyme inhibition. Later experiments by Karlmark *et al.* (1974), however, pointed out the possibility of a higher P_{CO_2} in the tubular fluid than in the arterial blood. This was found to be quantitatively equivalent to the chemical non-equilibrium proposed. From direct measurements with a P_{CO_2} microelectrode, Sohtell *et al.* (1976) obtained results which strongly favoured the assumption that the P_{CO_2} in the tubular fluid is higher than in the arterial blood.

The investigation was carried out in a multi-membrane system in which the hydrogen ion secretion and bicarbonate reabsorption were simulated by diffusion processes. It was shown that the rate of transport of the carbon dioxide could be altered.

Material and Methods

In order to study the reaction between H⁺ and HCO₃⁻ coming into a membrane from either side, the membrane was regarded as being divided into nine slices. A multi-chamber system was thus developed, in which the chambers were separated by cellophane membranes. The complete equipment (see Fig. 1) consisted of 10 thermostated chambers. The two end chambers were each connected to a glass container (3 litres) as reservoir I and II (Fig. 1) via a pump. The end chamber of the hydrogen side was No. 1 and that of the bicarbonate side No. 10. The membrane between chambers 1 and 2 was called membrane No. 1 and so forth. Stirring mechanisms driven by electromagnet and silver chloride electrodes for potential difference recordings were situated in the chambers (Fig. 1). In the present investigation the potential differences were recorded so as to be able to follow from outside the chambers the development of a steady state in the reaction system. The electrodes were connected to a mV meter (P11M26, Radiometer Copenhagen, Denmark). The experiments were performed at 25°C.

The membranes were cut from cellophane tubings (C 75 Dialysis membrane, Union Carbide Corporation, Chicago (Invents 60638)). When studying events taking place in membranes, the influence of the surrounding unstirred layers has to be considered. The effective layer connected to smooth cellophane membranes is not reduced below the order of 0.03 mm, even with vigorous stirring. The thickness of the unstirred layer

Thermostated waterbath

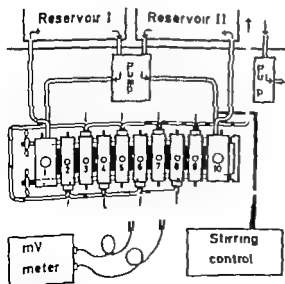
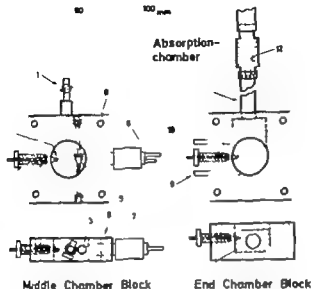


Fig. 1 The complete equipment showing chambers mounted on to a grade steel frame pressed together with wingnuts, the reservoirs in the thermostated water bath, the peristaltic pump circulating thermostated water and end blood solutions, respectively by the stirring electromagnets and mV-meter.



2 The chamber blocks: 1) stopcock, 3) Ag/AgCl electrode, 3) electrodeholder 4) O-rings, 11) as propeller, 13) stirrer and heater, 14) heater, 15) iron-core stirrer for thermostated percolate, 1) holes for the guide strand, 9) and 10) inlet and outlet for the circulating end-chamber solution, 11) acting tube, 12) container for the bicarbonate solution.

and therefore had to exceed 0.2 to 0.8 mm to minimize the influence of the unstirred layers (Teorell 1936). In investigation the membranes were made about 0.7 mm thick (wet thickness), through superposition of three pieces of the above-mentioned tubing (Teorell 1936).

The end chamber concentrations of H⁺ and HCO₃⁻ in this model experiment were so selected as to give pH values which are approximately comparable to the prevailing conditions in the proximal tubules of kidney 1. In order to evaluate the relative chemical forces (F) of the hydrogen ion secretion and the bicarbonate ion reabsorption, respectively in the proximal tubules, the following equation and values were used:

$$F = A \log \frac{C_{\text{secreted}}}{C_{\text{reabsorbed}}}$$

where A is a proportionality constant and C represents the concentration of the ion species. (H⁺) secreted = 10⁻⁶ M (Karlmark *et al.* 1974), cell [H⁺] = 10⁻⁷ M (Waddell *et al.* 1969). (HCO₃⁻) reabsorbed = 25 · 10⁻³ M (probably concentration of the glomerular filtrate in Bowman's space, without Donnan factor and plasma water concentration, the plasma concentration), cell [HCO₃⁻] = 11 · 10⁻³ M (Kjell *et al.* 1974). This resulted in a driving force for bicarbonate ions of about 0.35 and for hydrogen ions of 0.35.

In the first experiment the following solutions were deposited in the respective chambers. No. 1 100 mM NaCl, No. 2 9.5 mM NaCl and No. 10 95 mM NaHCO₃ + 5 mM NaCl. Chloride was placed in all chambers that potential changes could be followed from the beginning.

In the second experiment the catalytic effect of three kinds of macromolecules on the reaction system was studied. The macromolecules were dissolved in a 5 mM NaCl solution to equal final concentrations. The flowing solutions were studied: 0.05 mM bovine carbonic anhydrase type B (HCA B), 0.05 mM bovine serum albumin (AB Kabi, Stockholm, Sweden) and 0.05 mM dextran (Dextran T₅₀, Pharmacia Fine Chemicals, Uppsala, Sweden). These solutions were deposited in chambers 2-9. The concentration of carbonic anhydrase was about 10 times that in the kidney cell (Maren 1969).

Kindly received from Dr P. Wernstedt, Department of Pharmacology Uppsala University Uppsala, Sweden.

40 mmHg. During control conditions this difference was small. After carbonyl cyanide inhibition however these mean differences ranged between 0.41 to 0.85 pH units. These were interpreted as a chemical nonequilibrium of the bicarbonate buffer system, induced by the enzyme inhibition. Later experiments by Karlmark *et al* (1974), however pointed out the possibility of a higher P_{CO_2} in the tubular fluid than in the arterial blood. This was found to be quantitatively equivalent to the chemical non-equilibrium proposed. From direct measurements with a P_{CO_2} -microelectrode, Sohtell *et al* (1976) obtained results which also favoured the assumption that the P_{CO_2} in the tubular fluid is higher than in the arterial blood.

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The membranes were cut from cellophane tubing (C 75 Dialysis membrane, Union Carbide Corporation, Chicago, Illinois 60638). When studying events taking place in membranes, the influence of the surrounding unstirred layers has to be considered. The effect of a layer connected to smooth cellophane membranes is not reduced below the order of 0.03 mm, even with vigorous stirring. The thickness of the

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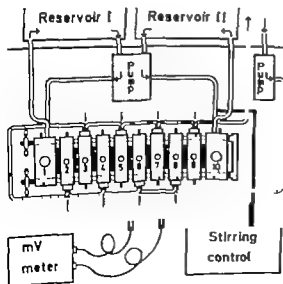
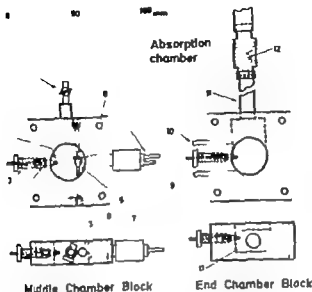


Fig. 1 The complete equipment showing the chambers mounted on a guide stand and pressed together with wingnuts, the two reservoirs in the thermostated water bath, the circulating thermostated water and solutions, respectively the stirring electromagnets and the mV meter.



2 The chamber blocks: 1) stepcock, 2) Ag/AgCl electrode, 3) electrodeholder, 4) O-rings, 5) propeller on its spider and bolt-on rod magnet, 6) iron-core with channels for thermostatted steel, 7) basin for the gas stand, 8) and 10) basin and outlet for the circulating end-chamber solution, 11) syringe tube, 12) connector for the hydrogen-dihydroxide solution.

is therefore had to exceed 0.2 to 0.8 atm to minimize the influence of the unstirred layers (Teorell 1936). In an experiment the membranes were made about 0.7 mm thick (wet thickness), through superimposition of four pieces of the borosiluminated tubings (Teorell 1934).

The end chamber concentrations of H^+ and HCO_3^- in the model experiment were so selected as to give an ionic relation are approximately comparable to the prevailing conditions in the proximal tubules of kidney in order to evaluate the relative chemical forces (F) of the hydrogen ion secretion and the membrane ion reabsorption, respectively in the proximal tubules, the following equation and values are

$$F = A \log \frac{C_{\text{inner}}}{C_{\text{out}}}$$

where A is proportionality constant and C represents the concentration of the ion species (H^+) known = 10^{-7} M (Karlmark *et al.* 1974), cell $8.9 \cdot 10^{-8}$ M (Waddell *et al.* 1969), (HCO_3^-) known = $2.5 \cdot 10^{-3}$ M, probable concentration of the glomerular filtrate in Bowman's space, 10^{-3} M (Dennis factor and plasma for correction, i.e. the plasma concentration), cell $11.1 \cdot 10^{-3}$ M (Kilbom *et al.* 1974). This resulted in an driving force for bicarbonate ions of about 0.35 and for hydrogen ions of -0.35 .

In the first experiment the following solutions were deposited in the respective chambers: No. 1 100 mM Tris, No. 2-4 5 mM NaCl and No. 10 95 mM $NaHCO_3$, 5 mM NaCl. Chloride was placed in all chambers that potential changes could be followed from the beginning.

In the second experiment the catalytic effect of three kinds of macromolecules on the reaction system was studied. The macromolecules are dissolved in 5 mM NaCl solution to equal final concentrations. The following solutions were studied: 0.1 mM human carbonic anhydrase type III (HCA), 0.05 mM bovine serum albumin (BSA, Kabi, Stockholm, Sweden) and 0.05 mM dextran (Dextran T₅₀, Pharmacia Fine Chemicals, Uppsala, Sweden). These solutions are deposited in chambers 2-9. The concentration of carbonic anhydrase is about 10 times that in the kidney cell (Maren 1969).

— Kindly received from Dr P. Wernstedt, Department of Pharmacology, Uppsala University, Uppsala, Sweden.

In the *third* experiment the solutions were the same as in the first and second expts. The NaHCO_3 to chambers 1 and 10 was studied as the rate of carbon dioxide liberation from these chambers. The released carbon dioxide was absorbed and titrated by a barium-dihydroxide solution with brom-thymol blue as an indicator. The solution was kept in a small cup situated in the absorption chamber. In Fig. 2.

Analyses of about 500 μl of the content of each chamber were performed by the following methods

- (H⁺) Hydrolytation with brom-thymol-blue as indicator performed in a micro-titration (Öbrink 1955).
 (Cl⁻) Micro-chloride titration according to the method of Ramsay (Ramsay *et al.* 1955).
 (Na⁺) Integrating flame photometry (Öberg *et al.* 1967).
 (HCO_3^-) Bicarbonate was analysed as the total carbonic acid content according to a modified Capps (Öbrink 1955).
 P_{CO_2} P_{CO_2} measurements were performed with a P_{CO_2} electrode (ES036, Radiometer Copenhagen, Denmark) connected to a mV-meter (PHM26, Radiometer).

Results

Fig. 3 shows the ion concentrations and the carbon dioxide tension profiles in the first experiment. The results are considered to have been obtained under a relatively steady condition (after 5 days). According to the carbon dioxide profiles the neutralisation reaction between H⁺ and HCO_3^- took place somewhere in the eighth membrane. The quotient between the P_{CO_2} gradients from the reaction centre will be equal to the P_{CO_2} quotient between the outermost membranes, as the beginnings of the gradients are the same. The P_{CO_2} in a membrane was calculated as the mean P_{CO_2} of the adjacent chambers. The P_{CO_2} in membranes 8 and 9 were 110 and 87 mmHg, respectively which will give a quotient of 7.9.

In the *second* experiment where chambers 2 to 9 contained solutions of HCAB, albumin and dextran, a good steady state was obtained. The P_{CO_2} at the reaction centre, the P_{CO_2}

mmHg

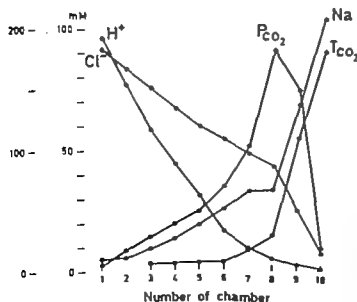
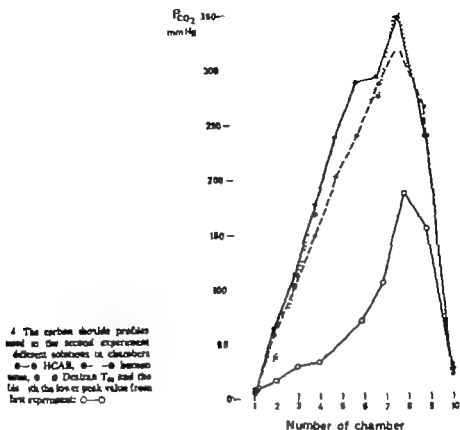


Fig. 3 The results in the first experiment obtained after a diffusion time of five days. T_{CO_2} represents the total carbonic acid content.



ies, are higher than in the first experiment (see Fig. 4). The quotients for the carbon dioxide gradients were 3.7, 5.0 and 4.0 for HCAE, albumin and dextran, respectively.

Table I shows the carbon dioxide liberation rates studied in the third experiment. The values have been normalized by setting the liberation rate from chamber No. 1 at 1 when chambers 2 to 9 contained 5 mM NaCl. The table also gives the quotients of the liberation rates from chambers 10 and 1 for the four middle chamber solutions examined.

TABLE I. The liberation rates of carbon dioxide and the quotients of the liberation rates between chambers 10 and 1 as obtained from the third experiment. These quotients are to be compared with the quotients for the carbon dioxide tension gradients in the first and second experiments.

Solutions	CO ₂ liberation, mm		
	No. 1	No. 10	Chamber No. 10/1
5 mM NaCl	1.0	9.3	9.3
5 mM NaCl + albumin	0.8	1.7	3.6
5 mM NaCl + HCAE	10.4	31.4	3.0
5 mM NaCl + dextran	7.5	20.3	2.7

In the *third* experiment the solutions were the same as in the first and second expts. The trapped carbon dioxide in chambers 1 and 10 was studied as the rate of carbon dioxide liberation from bacteria. The released carbon dioxide was absorbed and titrated by a barium-dihydroxide solution with thymol-blue as an indicator. The solution was kept in a small cup situated in the absorption chamber, as in Fig. 4.

Analyses of about 500 μ l of the content of each chamber were performed by the following method.

- (H⁺) Hydroxy titration with brom-thymol-blue as indicator performed in a micro-titration (Öbrink 1955).
- (Cl⁻) Micro-chloride titration according to the method of Ramsay (Ramsay *et al.* 1955).
- (Na⁺) Integrating flame photometry (Öberg *et al.* 1967).
- (HCO₃⁻) Bicarbonate was analysed as the total carbonic acid content according to a modified Com (Öbrink 1955).
- Pco₂ Pco₂ measurements were performed with a Pco₂ electrode (E5036, Radiometer Copenhagen, Denmark) connected to a mV-meter (PHM26, Radiometer).

Results

Fig. 3 shows the ion concentrations and the carbon dioxide tension profiles in the first experiment. The results are considered to have been obtained under a relatively steady state condition (after 5 days). According to the carbon dioxide profiles the neutralisation reaction between H⁺ and HCO₃⁻ took place somewhere in the eighth membrane. The quotient between the Pco₂ gradients from the reaction centre will be equal to the Pco₂ quotient between the outermost membranes, as the beginnings of the gradients are the same. The Pco₂ in a membrane was calculated as the mean Pco₂ of the adjacent chambers. The Pco₂ in membranes 8 and 9 were 11.0 and 87.2 mmHg, respectively which will give a quotient of 7.9.

In the *second* experiment, where chambers 2 to 9 contained solutions of HCAB, dextran, and dextran, a good steady state was obtained. The Pco₂ at the reaction centre, the p-

mmHg -

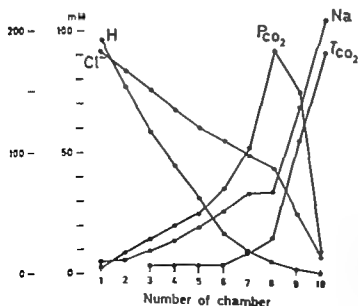
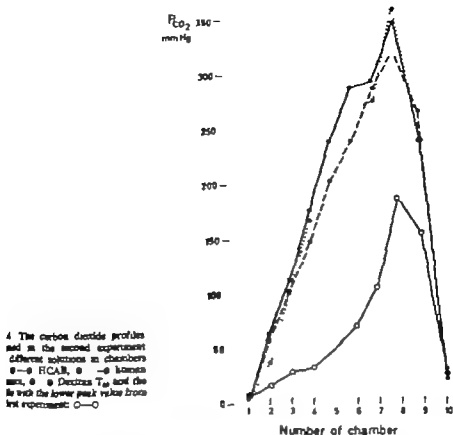


Fig. 3 The results in the first experiment obtained after a steady state of five days. Tco₂ represents the total carbonic acid content.



are higher than in the first experiment (see Fig. 4). The quotients for the carbon dioxide gradients were 3.7, 5.0 and 4.0 for HCAB, albumin and dextran, respectively. Table I shows the carbon dioxide liberation rates studied in the third experiment. The rates have been normalized by setting the liberation rate from chamber No. 1 at 1 when chambers 2 to 9 contained 5 mM NaCl. The table also gives the quotients of the liberation rates from chambers 10 and 1 for the four middle chamber solutions examined.

TABLE I. The liberation rates of carbon dioxide and the quotients of the liberation rates between chambers 10 and 1 as obtained from the third experiment. These quotients are to be compared with the quotients for the carbon dioxide tension gradients in the first and second experiments.

Solutions	CO ₂ liberation/min		
	No. 1	No. 10	Chamber No. 10/1
5 mM NaCl	1.0	9.3	9.3
5 mM NaCl albumin	6.0	21.7	3.6
5 mM NaCl HCAB	10.4	31.4	3.0
5 mM NaCl dextran	7.5	20.3	2.7

In the *third* experiment the solutions were the same as in the first and second expts. The NaHCO_3 to chambers 1 and 10 was studied as the rate of carbon dioxide liberation from these. The released carbon dioxide was absorbed and titrated by a barium-dihydroxide solution with thymol blue as an indicator. The solution was kept in a small cup situated in the absorption chamber. In Fig. 2.

Analyses of about 500 μl of the content of each chamber were performed by the following methods:

- (H⁺) Hydroxyl titration with brom-thymol-blue as indicator performed in a micro-titrator (Öbrink 1955).
 (Cl⁻) Micro-chloride titration according to the method of Ramsay (Ramsay *et al.* 1955).
 (Na⁺) Integrating flame photometry (Öberg *et al.* 1967).
 (HCO₃⁻) Bicarbonate was analysed as the total carbonic acid content according to a modified Cress (Öbrink 1955).
 P_{CO₂} P_{CO₂} measurements were performed with a P_{CO₂} electrode (E5036, Radiometer Copenhagen, Denmark) connected to a mV-meter (PHM 6, Radiometer).

Results

Fig. 3 shows the ion concentrations and the carbon dioxide tension profiles in the first experiment. The results are considered to have been obtained under a relatively steady state condition (after 5 days). According to the carbon dioxide profiles the neutralisation reaction between H⁺ and HCO₃⁻ took place somewhere in the eighth membrane. The quotient between the P_{CO₂} gradients from the reaction centre will be equal to the P_{CO₂} quotient between the outermost membranes, as the beginnings of the gradients are the same. The P_{CO₂} in air was calculated as the mean P_{CO₂} of the adjacent chambers. The P_{CO₂} in membrane 9 and 10 were 11.0 and 87.2 mmHg, respectively which will give a quotient of 7.9.

In the second experiment, where chambers 2 to 9 contained solutions of HCAB, agar and dextran, a good steady state was obtained. The P_{CO₂} at the reaction centre, the P_{CO₂}

mmHg —

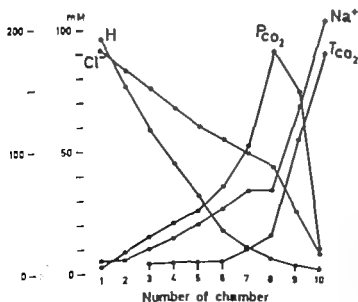
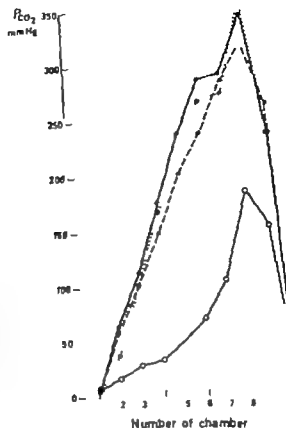


Fig. 3 The results in the first experiment obtained after a diffusion time of five days. T_{CO₂} represents the total carbonic acid content.



1. The carbon dioxide profiles used in the second experiment: different solutions in chambers: ●—● HCAB, ○—○ albumin sol., ●—● Dextran T₅₀ and the line with the lower peak value from first experiment: ○—○.

etc. were higher than in the first experiment (see Fig. 4). The quotients for the side gradients were 3.7, 5.0 and 4.0 for HCAB, albumin and dextran, respectively. Table I shows the carbon dioxide liberation rates studied in the *third* experiment. All have been normalized by setting the liberation rate from chamber No. 1 at 1; chambers 2 to 9 contained 5 mM NaCl. The table also gives the quotients of the $\frac{P_{CO_2}^{10} - P_{CO_2}^1}{P_{CO_2}^1 - P_{CO_2}^0}$ from chambers 10 and 1 for the four middle chamber solutions examined.

TABLE I. The liberation rates of carbon dioxide and the quotients of the liberation rates between chambers 10 and 1 as obtained from the third experiment. These quotients are to be compared with quotients for the carbon dioxide tension gradients in the first and second experiments.

Solutions	CO ₂ liberation/mm		Chamber No. 10/1
	No. 1	No. 10	
5 mM NaCl	1.0	9.3	9.3
5 mM NaCl albumin	6.0	21.7	3.6
5 mM NaCl HCAB	10.4	31.4	3.0
5 mM NaCl dextran	7.5	20.3	2.7

Discussion

In a system with several chambers in series, Teorell (1936) studied the distribution of ions across a membrane made up of the chamber-separating membranes. The ions were found to be distributed according to their mobilities and concentration differences in the chambers. He also studied the effect on ion distribution of a chemical reaction within the chamber system. From the distribution and the effects of concentration differences in the chamber system the location of the reaction can be calculated. The concentrations of hydrogen and bicarbonate ions are assumed to be equal in either end of the system. The relative mobilities of these ions are 350 and 43.5 respectively at 25°C (Handbook of Chemistry and Physics 1971). If the length of the system is set at 1.0 and the mobilities of the reacting ions are assumed not to be influenced by other ions, the reaction location will occur 0.11/1 of the distance from the side where bicarbonate emanates. In steady state the concentration profiles are linear to the point of reaction, which then means that the carbon dioxide concentration gradient and thus the rate of carbon dioxide transport towards the bicarbonate side is theoretically 8.0 times greater than that towards the hydrogen side. This value is in accordance with the result of 7.9 obtained in the first experiment, thus also verifying the asymmetrically located reaction centre.

A number of reports have described a facilitation of carbon dioxide transport through different boundaries. The concentrations of the reacting ions in the different chambers or compartments in the present investigation are considered to be homogenous, but not at the membranes and their connecting unstirred layers. Gutknecht *et al.* (1977) found that carbon dioxide diffusion can be rate limiting for total carbon dioxide transport in lipid bilayers and unstirred layers, as the neutralisation reaction is too slow to permit even a high bicarbonate concentration to speed up the carbon dioxide production more than 15%. In carbonic anhydrase-catalyzed reactions they found that the bicarbonate ion mobility in itself would be rate-limiting. This supports the finding of a lower peak P_{CO_2} in the case of the uncatalyzed reaction as compared with the catalyzed one, which thus could be an effect of a slow bicarbonate permeation through the unstirred layer. Suchdeo *et al.* (1974) found a 3 to 5 fold increase of the carbon dioxide flux through a Gelman membrane when adding carbonic anhydrase, and Enns (1967) reported a doubled transport rate through a Millipore filter washed red-cell ghosts or filtrate from the red-cell solution deposited on one side of the membrane.

The relative resistance to permeation of the reacting ions in the unstirred layers can be decreased by co-transport in translational diffusion of macromolecules. Gros *et al.* (1973) observed that thin layers of albumin solutions facilitated hydrogen ion diffusion and also found greater permeation of bicarbonate ions, which was explained as a secondary effect of the increased hydrogen ion diffusion. The facilitating effect of the dextran solution is not fully understood. The results from the second experiment, in which the macromolecular solutions were used, showed a tendency to enhanced transport of carbon dioxide towards the hydrogen and bicarbonate ion side, indicated by the increased P_{CO_2} gradients compared to the first experiment. This facilitation was demonstrated by the carbon dioxide flux study in the third experiment.

Recently Wistrand *et al.* (1977) showed the existence of an enzyme that is kinetically similar H⁺ATPase in the brush border and peritubular membranes of the rat kidney. The membrane μ ase was found to have 3% of the total cell enzyme activity. If the distribution of proteins in the cell is equal to that in the membranes, this should lead to a somewhat higher concentration in the membranes. According to Maren (1969), however, only 0.1% of the enzyme activity in the tubular tissue is utilized. The amount of carbonic anhydrase in the present preparation is therefore regarded as being in a suitable excess.

The increasing effect of carbonic anhydrase on the reaction rate is, however, of minor importance in the present system. As shown in Fig. 4, all the macromolecules studied give out the same carbon dioxide profile. This means that the macromolecular reduction of the distance to permeation of the reacting ions is of the same order. The permeation of the ions appears to be rate-limiting for carbon dioxide transport. The effect of the carbonic anhydrase on the reaction rate would probably have been more pronounced if the carbon dioxide transport had been rate-limiting in this membrane system. The driving force for carbon dioxide diffusion through a membrane should be increased by an enzymatic conversion of carbon dioxide into bicarbonate.

The phenomenologically described chemical course in this investigation on interacting hydrogen and bicarbonate ions could be analogous to the situation in the proximal tubules. The finding of the elevated P_{CO_2} in the proximal tubules (Soltoff *et al.* 1976) may be a result of a neutralization reaction taking place adjacent to the luminal cell membrane. The elevated pH in the reaction centre would create an increased luminal fluid P_{CO_2} and a build-up of a solvent for carbon dioxide transport out from the tubular lumen. The results also indicate that the net carbon dioxide transport is enhanced by carbonic anhydrase, by its increasing effect on the permeation of hydrogen and bicarbonate ions.

References

- WILKINSON, T. Facilitation by carbonic anhydrase of carbon dioxide transport. *Science* 1967 155 44-47.
- WILKINSON, T. C. and T. H. MAREN. The rates of hydration of carbon dioxide and dehydration of carbonic acid at 37°C. *Biochim. Biophys. Acta (Amst.)* 1972 261 70-76.
- WILKINSON, T. C. and W. MOLL. Facilitated diffusion of CO₂ across albumin solutions. *J. gen. Physiol.* 1974 64 359-371.
- YENIGUCHI, J., M. A. BUSTON and F. C. TARRANT. Diffusion of carbon dioxide through lipid bilayer membranes. Effect of carbonic anhydrase, bicarbonate and unstirred layers. *J. gen. Physiol.* 1977 69 779-794.
- ANDERSON, J. *Chemistry and Physics*, 51 Ed. 1971. The Chemical Rubber Co. Cleveland, Ohio.
- FAHREHART, B. and R. O. D. NELSON. Titration of P_{CO_2} , bicarbonate and ammonium ions along the rat proximal tubule. *Am. J. med. Sci.* 1978 91 243-258.
- WILKINSON, T. C., R. N. S. K. AGGARWAL, K. BOGAARTIAN, R. NABAR and W. WINE. Intracellular bicarbonate in single cells of rat kidney proximal tubules. *Pflügers Arch. ges. Physiol.* 1974 349 293-299.
- WILKINSON, T. H. Renal carbonic anhydrase and the pharmacology of sulfonamide inhibitors. *Handb. Exp. Pharm.* 1969 24 195-256.
- WILKINSON, T. H. R. LYNN and B. WALLIN. A integrating flame photometer for simultaneous micro-analysis of sodium and potassium in biological fluids. *Analyt. Biochem.* 1967 18 543-558.
- WILKINSON, K. S. A modified Cornay unit for microdiffusion analysis. *Biochem. J.* 1955 59 1-134.
- WILKINSON, J. A., R. H. J. BROWN and P. C. CHODMAN. Electrostatic titration of chloride in small volumes. *J. Chem. Soc. Faraday* 1955 51 822-829.
- WILKINSON, T. C., N. W. CARTER and D. W. SELDREY. With the technical assistance of A. C. NUNO. The mechanism

- ism of bicarbonate reabsorption in the proximal and distal tubules of the kidney *J. clin. Invest.* 44: 2, 278-290.
- SCHTELL, M. and B. KARLMARK, In vivo micropuncture Pco_2 measurements. *Pflügers Arch. ges. Ph.* 1976. 363: 179-180.
- SUCHTELO, S. R. and J. S. SCHULTZ, Mass transfer of CO_2 across membranes: facilitation by the peroxo-bicarbonate ion and of the enzyme carbonic anhydrase. *Biochem. biophys. Acta (Amst.)* 1974. 35: 1-440.
- TEORELL, T. A method of studying conditions within diffusion layers. *J. biol. Chem.* 1936. 113: 735-74.
- WADDELL, W. J. and R. M. BATES, Intracellular pH. *Ph. siol. Rev.* 1969. 49: 285-329.
- VIEIRA, F. C. and G. MALNOC, Hydrogen ion secretion by rat renal cortical tubules as studied by an intra-microelectrode. *Amer. J. Physiol.* 1968. 214: 710-718.
- WISTRAND, P. J. and R. KINNE, Carbonic anhydrase activity of isolated brush border and basolateral membranes of renal tubular cells. *Pflügers Arch. ges. Physiol.* 1972. 370: 1-126.

P_{CO_2} of the proximal tubular fluid and the efferent arteriolar blood in the rat kidney

By

MORGAN SÖRTÉLL

Received 25 April 1978

Abstract

SÖRTÉLL, M. P_{CO_2} of the proximal tubular fluid and the efferent arteriolar blood in the rat kidney. Acta physiol. scand. 1979 105: 137-145.

Measurements *in vivo* of the carbon dioxide tension of the proximal tubular fluid and of the efferent arteriolar blood were performed with P_{CO_2} microelectrodes in the rat kidney. The buffer lines of the efferent arteriolar and of systemic arterial blood were determined with an ultramicro equilibration system and the acid-base status of the systemic arterial blood was measured. The intratubular P_{CO_2} was significantly higher than P_{CO_2} of the arterial blood, and the P_{CO_2} of the efferent arteriolar blood was significantly lower than that of the arterial blood. The buffer capacity was higher and the bicarbonate concentration slightly lower for efferent arteriolar blood than for the arterial blood. It is concluded that P_{CO_2} difference exists across the tubular wall and that the high intratubular P_{CO_2} favours chemical equilibrium of the carbonic acid-bicarbonate system in the proximal tubular fluid. It is supposed that the slightly lowered bicarbonate concentration in the efferent arteriolar blood is an effect of the glomerular ultrafiltration process.

During the ultrafiltration process in the kidney the proximal tubules are offered a load of bicarbonate. The mechanisms underlying the tubular bicarbonate reabsorption have been the subject of much discussion (see Malnic and Giebisch 1976). One of these mechanisms generally favoured is the active hydrogen ion transport into the tubular lumen. According to this hypothesis the H^+ and HCO_3^- ions react with one another and form carbonic acid. This acid is then dehydrated, forming water and carbon dioxide, which are then reabsorbed. The rate of this reabsorption may be influenced by various factors, such as the rate of delivery of H^+ , the hydration rate and the carbon dioxide diffusion across the tubular wall.

Rector *et al* (1965) and Vachra and Malnic (1968) made direct recordings of the intratubular pH with microprobe techniques. They measured the pH of urine samples taken from the same puncture site and equilibrated to a P_{CO_2} of 40 mmHg, and found the *in situ* pH to be lower than the pH of the equilibrated samples. It was concluded that the lower *in situ* pH was due to a lower rate of dehydration of the carbonic acid, and it was thus considered to represent a chemical non-equilibrium state.

Karlmark and Danneberg (1974) also made *in situ* pH measurements but equilibrated the urine samples from the proximal tubules to CO_2 of different concentrations. They were then

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- SÖHTELL, M. and B. KARLMARK. In vivo micropuncture Pco_2 measurements. *Pflügers Arch. ges. Physiol.* 1976 363 179-180.
- SUCHDEO, S. R. and J. S. SCHULTZ. Mass transfer of CO_2 across membranes: facilitation by bicarbonate ion and of the enzyme carbonic anhydrase. *Biochem. Biophys. Acta (Amst.)* 1974 41 440.
- TROSKELL, T. A method of studying conditions within diffusion layers. *J. Biol. Chem.* 1936 113 735-7.
- WADDELL, W. J. and R. G. BATES. Intracellular pH. *Physiol. Rev.* 1969 49 285-329.
- VEIRA, F. C. and B. MALM. Hydrogen ion secretion by rat renal cortical tubules as studied by a new microelectrode. *Amer. J. Physiol.* 1968. 214 710-718.
- WETTERSTRAND, P. J. and R. KINNE. Carbonic anhydrase activity of isolated brush border and basolateral membranes of renal tubular cells. *Pflügers Arch. ges. Physiol.* 1972. 370. 121-126.

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In the ultrafiltration process in the kidney the proximal tubules are offered a load of bicarbonate. The mechanisms underlying the tubular bicarbonate reabsorption have been the subject of much discussion (see Malmik and Giebisch 1976). One of these mechanisms generally favoured is the active hydrogen ion transport into the tubular lumen. According to this hypothesis the H^+ and HCO_3^- ions react with one another and form carbonic acid. This acid is then hydrated, forming water and carbon dioxide, which are then reabsorbed. The rate of this absorption may be influenced by various factors, such as the rate of delivery of H^+ , the hydration rate and the carbon dioxide diffusion across the tubular wall.

Rector *et al.* (1963) and Vieira and Malmik (1968) made direct recordings of the intratubular pH with microprobe techniques. They measured the pH of urine samples taken from the same puncture site and equilibrated to P_{CO_2} of 40 mmHg, and found the *in situ* pH to be lower than the pH of the equilibrated samples. It was concluded that the lower *in situ* pH was due to a lower rate of dehydration of the carbonic acid, and it was thus considered to represent a chemical non-equilibrium state.

Karlmark and Danielsson (1974) also made *in situ* pH measurements but equilibrated the urine samples from the proximal tubules to CO_2 of different concentrations. They were then

able to calculate the actual P_{CO_2} of the tubular urine. This was found to be higher than P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with respect to the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} at the tubular membrane.

With the aim of contributing to the discussion of whether the dehydration represents chemical non-equilibrium or equilibrium state, direct P_{CO_2} measurements were made with P_{CO_2} microelectrodes in this investigation.

With the technique used it was also possible to evaluate the acid-base conditions of blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between proximal tubular urine and the blood of the early peritubular capillaries.

Material and Methods

Male albino rats (Sprague Dawley strain A litter, Stockholm, Sweden) weighing 225 to 325 g were kept on a diet of commercial rat pellets (Anticimex, Sweden) and were allowed food and water *ad libitum* until the start of the experiment. They were anesthetized by an intraperitoneal injection of Inactin (Karl Faber Promonta GmbH Hamburg, W. Germany); a dose of 120 mg/kg body weight (b.w.).

The body temperature was kept at 37.5°C with a servo-controlled heating table. The rats were tracheotomized and a catheter was inserted into the left femoral vein and left femoral artery for infusions and pressure recording, respectively. After catheterization a continuous intravenous (i.v.) infusion of 3 ml b.w. per hour of a Ringer solution was started. The urinary bladder was drained to avoid distension of flow (Karlmark and Danielson 1974). The left kidney was reached through a flank incision and was immobilized in a lock cup by embedding it in 2% agar-Ringer solution. The left ureter was catheterized and the exposed dorsal surface of the kidney was superfused with warm liquid paraffin, pre-equilibrated with a mixture of 5% CO_2 and 95% O_2 in nitrogen.

Experimental procedure. For the biological experiments the rats were divided into two groups.

In group I the P_{CO_2} microelectrode was used for *in situ* determinations of the carbon dioxide tension in the convoluted proximal tubules and in the superficial efferent arterioles (welling points). The proximal tubules investigated were chosen randomly. Arterial blood was sampled simultaneously for determination of the acid-base status according to the method of Siggard Andersen (1967).

The rats of group II were used for measurement of the acid-base condition of welling point blood in a micro technique described below. 15 min before the blood was collected, the rats were given an infusion of 2 000 IU/kg b.w. of a heparin solution (Vitrum, Stockholm, Sweden) to avoid clotting of the blood. Sampling pipettes were pulled and ground in a similar manner to the outermost capillary of the P_{CO_2} microelectrode (see below). The tip was given a diameter of about 12 μ m. The sampling pipette was filled with coloured liquid paraffin. The blood flowed into the pipette by its own pressure. Arterial blood was collected by analysis in the Astrup apparatus.

The P_{CO_2} microelectrode. The main principle of this electrode is well known (Savermogren and Sjöblom 1958). It has previously been described by Sohtell and Karlmark (1976) and brief description is given as follows: A ordinary Pyrex glass tube (inner diameter 1.0 mm, outer diameter 1.4 mm) is pulled to a diameter of a few μ m and ground angularly to permit gentle puncture. An antimony-Ag/AgCl electrode system is made and introduced together with a second capillary into the ground glass tube, as shown in Fig. 1. A third glass capillary is mounted in the rear end and all components are glued together. The liquid electrodes leave the unit through the glue drop. A solution containing 0.145 M NaCl and 5 mM NaHCO₃ is aspirated into the electrode through the tip. The electrode potential is then measured and must have an empirically accepted value, i.e. 300 ± 15 mV. The tip is finally plugged by dipping it into a drop of clear rubber (Elastosil, Wacker-Chemie, GmbH, München, W. Germany). This special rubber is used because it requires moisture for curing, which assures a tight plug.

The electrode is calibrated before and after each measurement. The device designed as a block of cups with nozzles in the bottom through which high gas flows give well-defined pressures of gas. The calibration cups are shown in Fig. 2 close to kidney cup. The calibration gas is thermostated to the same temperature as that of the tissue. The calibration and measurement period of three minutes respectively according to Sohtell and Karlmark (1976).

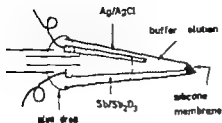


Fig. 1

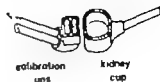


Fig. 2

1 The P_{CO_2} electrode. The three glass capillaries are connected to each other as well as to the pH electrode system by the same drop of glass (spontaneous).

2 The thermostated calibration cups and the kidney cup as arranged for tissue studies.

Arterial gases of the rat. This is determined as the acid-base status of the arterial blood by the Astrup calibration technique as described by Siggard Andersen (1967), using the nomogram for man, which can be used for animals too. The normal values, compared to man, are different, however.

Determination of the buffer lines of all blood samples. The analyses were performed in an equilibration device coded by Karlmark and Sobell (1977). This consists of closed thermostated air jacket surrounding 8 beakers containing liquid paraffin. The liquid paraffin in each beaker can be equilibrated individually to gas mixtures through a nozzle in the bottom of the beaker. The antimony electrode system used for the 1 measurement originally described is exchanged, however for glass-cup macroelectrode (Karlmark *et al.* 1971). A schematic drawing of the electrode arrangement is given in Fig. 3. The electrode is calibrated in phosphate buffer solutions in a beaker containing water-saturated liquid equilibrated with air.

The electrode can then be transferred to a beaker containing an equilibration gas mixture of 3% CO_2 in oxygen. A 10 to 15 μ l blood sample is deposited on the glass-cup macroelectrode and the sample droplet is isolated with Ag/AgCl reference electrode. The pH is measured and the procedure is then repeated in air control equilibrated with 12% CO_2 in oxygen.

1 The present investigation the equilibration microelectrode method was standardized with the arterial blood calibrated in the Astrup apparatus. The buffer lines of the arterial blood obtained with the two methods are parallel. These obtained with the micro-electrode, however, about 0.04 pH units more acid than the ones from the Astrup apparatus. This is probably due to protein error of the glass-cup macroelectrode in an equilibration micro-electrode.

The values of the buffer lines were evaluated in the Siggard Andersen nomogram as follows. The relation between the buffer lines of the blood point and arterial blood was calculated after the analyses of blood in an ultramicro equilibration unit. This relation was transferred to the buffer line of the arterial blood, analyzed in the Astrup apparatus and plotted in the nomogram. A true representation of the buffer lines is thus obtained.

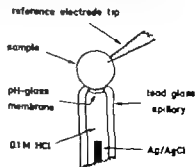


Fig. 3. The tips of the electrodes as arranged in the equilibration microelectrode unit. The drawing also shows sample droplet on the pH-glass membrane designed as a cup.

able to calculate the actual P_{CO_2} of the tubular urine. This was found to be higher than the P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} in the tubular membrane.

With the aim of contributing to the discussion of whether the dehydration represents a chemical non-equilibrium or equilibrium state, direct P_{CO_2} measurements were made with P_{CO_2} microelectrodes in this investigation.

With the technique used it was also possible to evaluate the acid-base conditions of the blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between proximal tubular urine and the blood of the early peritubular capillaries.

Material and Methods

Male albino rats (Sprague-Dawley strain, Anticimex, Stockholm, Sweden) weighing 225 to 325 g were fed a diet of commercial rat pellets (Anticimex, Sweden) and were allowed food and water *ad libitum* until the start of the experiment. They were anesthetized by an intraperitoneal injection of Inactin (ICF, F. Hoffmann-Laurig, GmbH, Hamburg, W. Germany) in a dose of 170 mg/kg body weight (b.w.).

The body temperature was kept at 37.5°C with a servo-controlled heating table. The rats were tracheotomized and a catheter was inserted into the left femoral vein and left femoral artery for infusions and blood pressure recording, respectively. After canneterization a continuous intravenous (i.v.) infusion of 3.0 ml b.w. and hour of Ringer solution was started. The urinary bladder was drained to an open drainage system (Karlmark and Danneberg 1974). The left kidney was reached through a flank incision and was immobilized in a Lucite cup by embedding it in a 1% agar-in-Ringer solution. The left ureter was cut and the exposed dorsal surface of the kidney was superfused with warm liquid paraffin, pre-equilibrated with a mixture of 5% CO_2 and 20% O_2 in nitrogen.

Experimental procedure. For the biological experiments the rats were divided into two groups.

Group I: The P_{CO_2} microelectrode was used for *in vivo* determinations of the carbon dioxide tension in the cornuoluted proximal tubules and in the superficial efferent arterioles (welling points). The proximal tubules investigated were chosen randomly. Arterial blood was sampled simultaneously for determination of the acid-base status according to the method of Siggaard Andersen (1967).

The rats of group II were used for measurement of the acid-base condition of the glomerular blood with a micro technique described below. 15 min before the blood was collected, the rats were given an i.v. injection of 0.001 IU/kg b.w. of a heparin solution (Vetrum, Stockholm, Sweden) to avoid clotting of the blood. Sampling pipettes were pulled and ground in a similar manner to the outermost capillary of the P_{CO_2} microelectrode (see below). The tip was given a diameter of about 12 μm . The sampling pipette was filled with coloured liquid paraffin. The blood flowed into the pipette by its own pressure. Arterial blood was collected for analyses in the Astrup apparatus.

The P_{CO_2} microelectrode. The main principle of this electrode is well known (Sjeverghaus and Brä 1958). It has previously been described by Schtell and Karlmark (1976) and a brief description is given in the following. An ordinary Pyrex glass tube (inner diameter 1.0 mm, outer diameter 1.4 mm) is pulled to a diameter of a few μm and ground angularly to permit a gentle puncture. An antimony Ag/AgCl electrode system is made and introduced together with a second capillary into the ground glass tube, as shown in Fig. 1. A third glass capillary is mounted in the rear end and all components are glued together. The CO_2 electrodes leave the unit through the glue drop. A solution containing 145 mM NaCl and 5 mM NaHCO₃ is aspirated into the electrode through the tip. The electrode potential is then measured and must be empirically accepted value, i.e. 500 ± 15 mV. The tip is finally plugged by dipping it into a drop of sticky rubber (Elastoll, Wacker-Chemie, GmbH, München, W. Germany). This special rubber is used because it requires moisture for curing, which assures a tight plug.

The electrode is calibrated before and after each measurement in a device designed as block of cups with nozzles in the bottom through which high gas flows give well-defined pressures of carbon dioxide. The calibration cups are shown in Fig. 2 close to kidney cup. The calibration gas is humidified at the same temperature as that of the tissue. The calibration and measuring time is after a period of three minutes respectively according to Schtell and Karlmark (1976).

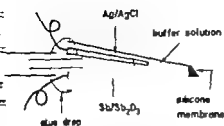


Fig. 1

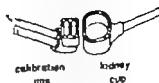


Fig. 2

1 The P_{CO_2} electrode. The three glass capillaries are connected to each other as well as to the pH probe (which by the same drop of glue (epoxy resin)).

The thermostated calibration cups and the kidney cup as arranged for these studies.

Verification of the pH This was determined as the acid-base status of the arterial blood by the Astrup technique as described by Siggard Andersen (1967), using the nomogram for man, which can also be used for animals too. The normal values, compared to man, are different, however.

Determination of the buffer line of all blood samples. The analyses were performed in an equilibration device described by Karlmark and Sobel (1973). This consists of closed thermostated steel jacket surrounding six beakers containing liquid paraffin. The liquid paraffin in each beaker can be equilibrated individually with gas mixture through a nozzle at the bottom of the beaker. The potentiometry electrode system used for the measurements originally described was changed, however, for glass-cup microelectrode (Karlmark et al. 1971). A schematic drawing of the electrode arrangement is given in Fig. 3. The electrode is calibrated with phosphate buffer solutions in a beaker containing water-saturated liquid equilibrated with air.

The electrode was then transferred to a beaker containing an equilibration gas mixture of 3% CO_2 in oxygen. A 10 to 15 μ l blood sample is deposited on the glass-cup microelectrode and the sample droplet is secured with Ag/AgCl reference electrode. The pH is measured and the procedure is then repeated in water-saturated liquid equilibrated with 1% CO_2 in oxygen.

In the present investigation the equilibration ultramicro method was standardized with the arterial blood collected in the Astrup apparatus. The buffer lines of the arterial blood obtained with the two methods are parallel. Those obtained with the micro method were, however, about 0.04 pH units more acid than the ones from the Astrup apparatus. This is probably due to a positive error of the glass-cup microelectrode in the equilibration micro-test.

The shifts of the buffer lines were evaluated in the Siggard Andersen nomogram as follows. The relation between the buffer lines of arterial blood and arterial blood was calculated after the analysis of blood in the ultramicro equilibration unit. This relation was transferred to the buffer line of the arterial blood, related to the Astrup apparatus and plotted in the nomogram. A true representation of the buffer lines can then be obtained.

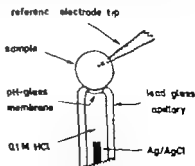


Fig. 3 The type of the electrodes as arranged in the equilibration ultramicro test. The drawing also shows sample droplet on the pH-glass membrane designed as a cup.

able to calculate the actual P_{CO_2} of the tubular urine. This was found to be higher than the P_{CO_2} of the arterial blood and they concluded that a chemical equilibrium existed with respect to the dehydration of the carbonic acid, but a non-equilibrium concentration of P_{CO_2} in the tubular membrane.

With the aim of contributing to the discussion of whether the dehydration represents a chemical non-equilibrium or equilibrium state, direct P_{CO_2} measurements were made with P_{CO_2} microelectrodes in this investigation.

With the technique used it was also possible to evaluate the acid-base conditions of the blood of the efferent arterioles and thus calculate the difference in P_{CO_2} between proximal tubular urine and the blood of the early peritubular capillaries.

Material and Methods

Male albino rats (Sprague Dawley strain Anticomer, Stockholm, Sweden) weighing 225 to 325 g were fed a diet of commercial rat pellets (Anticomer, Sweden) and were allowed food and water *ad libitum* until the start of the experiment. They were anaesthetized by an intraperitoneal injection of letobarbital (Fa. Promonta GmbH Hamburg, W. Germany) in a dose of 170 mg/kg body weight (b.w.).

The body temperature was kept at 37.5°C with a servo-controlled heating table. The rats were tracheotomized and a catheter was inserted into the left femoral vein and left femoral artery for infusions and blood pressure recording, respectively. After catheterization a continuous intravenous (i.v.) infusion of 3 ml b.w. per hour of a Ringer solution was started. The urinary bladder was drained to avoid distension (Karlmark and Danielson 1974). The left kidney was reached through a flank incision and was immobilized in a lucite cup by embedding it in a paraffin-Ringer solution. The left ureter was catheterized and the exposed dorsal surface of the kidney was superfused with warm liquid paraffin, pre-equilibrated with a mixture of 5% CO_2 and 95% O_2 in nitrogen.

Experimental procedure. For the biological experiments the rats were divided into two groups. In group I the P_{CO_2} microelectrode was used for several determinations of the carbon dioxide tension in the convoluted proximal tubules and the superficial efferent arterioles (welling points). The proximal tubules investigated were chosen randomly. Arterial blood was sampled simultaneously for determination of the acid-base status according to the method of Siggaard Andersen (1967).

The rats of group II were used for measurement of the acid-base condition of welling point blood. At the micro technique described below 15 min before the blood was collected the rats were given an i.v. infusion of 2 000 IU/kg b.w. of a heparin solution (Vitrum, Stockholm, Sweden) to avoid clotting of the blood. Sampling pipettes were pulled and ground in a similar manner to the outermost capillary of the P_{CO_2} microelectrode (see below). The tip was given a diameter of about 12 μm . The sampling pipette was filled with coloured liquid paraffin. The blood flowed into the pipette by its own pressure. Arterial blood was collected for analyses in the Astrup apparatus.

The P_{CO_2} microelectrode. The main principle of this electrode is well known (Severinghaus and Slikk 1958). It has previously been described by Sohtell and Karlmark (1976) and brief description is given in the following. A ordinary Pyrex glass tube (inner diameter 1.0 mm, outer diameter 1.4 mm) is pulled to a diameter of a few μm and ground angularly to permit a gentle puncture. An aluminium-mercury system is made and introduced together with a second capillary into the ground glass. A third glass capillary is mounted in the rear end and all components are glued together. The electrodes leave the unit through the glue drop. A solution containing 145 mM NaCl and O_2 is aspirated into the electrode through the tip. The electrode potential is then measured and empirically accepted value, i.e. 500 ± 15 mV. The tip is finally plugged by dropping it into a rubber (Elastosil, Wacker-Chemie, GmbH, München W. Germany). This special rubber is very hard and requires moisture for curing, which assures a tight plug.

The electrode is calibrated before and after each measurement in a device designed as a bicarbonate cups with nozzles in the bottom through which high gas flows give well-defined pressures of carbon dioxide. The calibration cups are shown in Fig. 2 close to kidney cup. The calibration gas is humidified and thermostated to the same temperature as that of the tissue. The calibration and measuring time is a period of three minutes respectively according to Sohtell and Karlmark (1976).

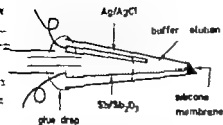


Fig. 1



Fig. 2

- 1 The P_{CO_2} electrode. The three glass capillaries are connected to each other as well as to the pH rods given by the same drop of glue (epoxy resin)
- 2 The thermostated calibration cups and the kidney cap as arranged for these studies.

acid-base status of the rat. This was determined as the acid-base status of the arterial blood by the Astrup titration technique as described by Siggard Andersen (1967), using the nomogram for man, which can be used for animals too. The normal values, compared to man, are different, however.

Measurement of the buffer base of rat blood samples. The analyses were performed in an equilibration device described by Karlmark and Soltefl (1973). This consists of a closed thermostated water jacket surrounding 2 beakers containing liquid paraffin. The liquid paraffin in each beaker can be equilibrated individually with a gas mixture through a needle in the bottom of the beaker. The antimony electrode system used for the measurements originally described was exchanged, however for a glass-cup microelectrode (Karlmark 1971). A schematic drawing of the electrode arrangement is given in Fig. 3. The electrode is calibrated in phosphate buffer solutions in a beaker containing water-saturated liquid equilibrated with air. The electrode is then transferred to a beaker containing an equilibration gas mixture of 3% CO_2 in gas A III to 15 ml blood sample deposited on the glass-cup microelectrode and the sample droplet is sealed with a Ag/AgCl reference electrode. The pH is measured and the procedure is then repeated in a similar constant equilibrated with 12% CO_2 in oxygen.

In the present investigation this equilibration microelectrode method was standardized with the arterial blood obtained in the Astrup apparatus. The buffer base of the arterial blood obtained with the two methods are parallel. Those obtained with the microelectrode were, however, about 0.04 pH units more acid than the values from the Astrup apparatus. This is probably due to a protein error of the glass-cup microelectrode in a calibration micro-scan.

The effect of the buffer base on the Siggard Andersen nomogram is shown in Fig. 4. The relation between the buffer base of arterial blood and arterial blood was calculated after the analysis of blood in a microelectrode equilibration scan. This relation was transferred to the buffer base of the arterial blood, which was the Astrup apparatus and plotted on the nomogram. A true representation of the buffer base is then obtained.

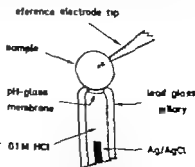


Fig. 3 The type of the electrodes as arranged in the equilibration microelectrode. The diagram also shows a sample droplet on the pH-glass membrane designed as a cup.

TABLE I. The acid-base parameters from the first group of rats. AB=arterial blood, WP =venous blood, TUB=tubular fluid. As=Astrup apparatus. El=microelectrode.

Rat	Sample	pH	Pco ₂ (As) mmHg	HCO ⁻ mM	Base excess mM	Pco ₂ (El) mmHg
1	AB 1	7.39	40.0	23.9	-0.2	
	TUB 1					65.7
	WP 1					39.3
	TUB 2					53.5
	TUB 3					61.1
	TUB 4					56.6
	TUB 5					64.2
	TUB 6					62.3
	WP 2					38.3
	WP 3					40.1
	TUB 7					43.2
	WP 4					40.2
	AB 1					
	TUB 1					40.4
	TUB 2					42.4
	TUB 3					46.1
3	AB 1	7.44	36.2	23.8	+0.6	
	WP 1					28.5
	TUB 1					74.5
	WP 2					27.4
	TUB 2					41.9
	TUB 3					77.6
	TUB 4					84.9
	TUB 5					82.5
	WP 3					29.0
4	AB 1	7.50	36.1	28.0	+5.1	
	TUB 1					63.7
	TUB 2					51.2
	TUB 3					48.1
	TUB 4					61.1
	TUB 5					57.4
	TUB 6					64.2
	WP 1					30.8
	TUB 7					42.4
	TUB 8					51.8
5	AB 1	7.43	34.6	22.8	-0.9	
	TUB 1					44.0
	WP 1					30.0
	TUB 2					55.4
	TUB 3					51.8
	TUB 4					64.7
	TUB 5					61.1
	TUB 6					78.7
	TUB 7					74.5
	TUB 8					81.8
	TUB 9					79.7

Results

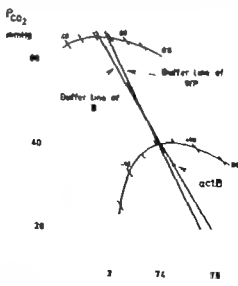
Acid-base status and Pco₂ The values for the acid base parameters of the arterial blood in the first group of rats are given in Table I. Fig. 4 shows the Pco₂ values in the proximal tubular lumen and in the walling points with the microelectrode.



4 The results of the P_{CO_2} measurements in the rats of group I. A: arterial blood, WP: welling point blood.

the tubular urine was always higher than that of the arterial blood taken in connection with the tubular measurements ($P < 0.001$).

In four of the five rats the P_{CO_2} measurements in the welling points were successful. These showed a slightly lower P_{CO_2} in the welling point blood than in the arterial blood ($P < 0.01$) buffer lines. In the second group of rats, the welling point blood and arterial blood were compared with respect to their buffer properties. The Siggaard Andersen nomogram for



5 The buffer lines obtained from rat 2 in group II. The arterial pH and the corresponding P_{CO_2} obtained for the arterial blood are shown on the buffer line by the point actB. A: arterial blood, WP: welling point blood, respectively. B: and BE are the scales in mM concentrations for buffer bases and base excess, respectively. The diagram is redrawn from the Siggaard Andersen nomogram (Radiometer Copenhagen, Denmark).

TABLE I The acid-base parameters from the first group of rats. AB = arterial blood, WP = walling blood, TUB = tubular fluid, As = Astrup apparatus, El = microelectrode.

Rat	Sample	pH	Pco ₂ (As) mmHg	HCO ₃ ⁻ mM	Base excess mM	Pco ₂ (El) mmHg
1	AB 1	7.39	40.0	23.9	-0.2	
	TUB 1					65.7
	WP 1					39.3
	TUB 2					53.5
	TUB 3					61.1
	TUB 4					56.6
	TUB 5					64.2
	TUB 6					62.3
	WP 2					38.3
	WP 3					40.1
	TUB 7					53.2
	WP 4					40.2
2	AB 1	7.49	27.0	20.8	-1.0	
	TUB 1					40.4
	TUB 2					42.4
	TUB 3					46.1
3	AB 1	7.44	36.4	23.8	+0.6	
	WP 1					28.5
	TUB 1					74.5
	WP 2					27.4
	TUB 2					41.9
	TUB 3					77.6
	TUB 4					84.9
	TUB 5					82.5
	WP 3					29.0
4	AB 1	7.50	36.1	28.0	+5.1	
	TUB 1					63.7
	TUB 2					51.2
	TUB 3					48.1
	TUB 4					61.1
	TUB 5					57.4
	TUB 6					64.2
	WP 1					30.8
	TUB 7					42.4
	TUB 8					51.8
5	AB 1	7.43	34.6	-2.8	-0.9	
	TUB 1					44.0
	WP 1					30.0
	TUB 2					55.4
	TUB 2					51.8
	TUB 4					64.7
	TUB 5					61.1
	TUB 6					78.7
	TUB 7					74.5
	TUB 8					81.8
	TUB 9					79.7

Results

Acid-base status and Pco₂ The values for the acid base parameters of the arterial blood obtained in the first group of rats are given in Table I. Fig. 4 shows the Pco₂ values obtained in the proximal tubular lumen and in the walling points with the Pco₂ microelectrodes. The Pco₂



Fig. 4 The results of the P_{CO_2} measurements in the rats of group I. AB = arterial blood, WP = welling point blood.

the tubular urine was always higher than that of the arterial blood taken in connection with the tubular measurements ($P < 0.001$).

In four of the five rats the P_{CO_2} measurements in the welling points were successful. These showed a slightly lower P_{CO_2} in the welling point blood than in the arterial blood ($P < 0.01$) buffer lines. In the second group of rats, the welling point blood and arterial blood were assessed with respect to their buffer properties. The Siggaard Andersen nomogram (or

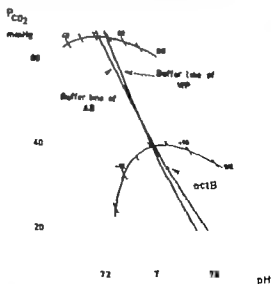


Fig. 5 The buffer lines obtained from rat in group II. The actual pH and the corresponding P_{CO_2} obtained for the renal blood are shown on the buffer line by the point AB. AB and WP are the arterial and welling point blood, respectively. BB and BE are the scales on the nomogram for buffer base and base excess, respectively. The diagram is taken from the Siggaard Andersen nomogram (Rasthæder Copenhagen, Denmark).

TABLE II The results of the measurements in group II AB = arterial blood, WP = welling point blood

Rat	Sample	pH	Pco ₂ mmHg	Actual HCO ₃ ⁻ mM	Base excess mM	log Pco ₂ /pH	Cross over point Pco ₂ /pH
1	AB 1	7.45	35.4	24.0	+0.7	-1.41	76.57.48
	WP 1					-1.50	
2	AB 1	7.43	35.0	22.8	-0.5	-1.57	76.57.38
	WP 1					-1.06	
3	AB 1	7.41	34.8	17	-1.9	-1.52	46.0.730
	WP 1					-2.27	
4	AB 1	7.46	36.6	3.7	+2.3	-1.48	38.0.749
	WP 1					-1.98	
	AB 2	7.49	29.7	22.2	+0.6	-1.59	46.3.7307
	WP 2					-1.00	
5	AB 1	7.47	34.3	3.4	+1.4	-1.79	55.6.730
	WP 1					-2.04	
	AB 2	7.46	34.0	23.8	+1.4	-1.73	50.7.307
	WP					-2.10	

man was used to evaluate the acid-base parameters. Fig. 5 shows the buffer line of arterial blood and of blood from a welling point at one measurement. The former line was obtained by the Astrup technique and the latter by the equilibration ultramicro technique. In all cases the absolute value of the slope (log Pco₂/pH) of the buffer line for welling point blood is greater than that for the arterial blood ($P < 0.01$) as shown in Table II which means that the welling point blood had a higher buffer capacity. In Fig. 6 the calculated mean values of the acid-base parameters of the welling point and arterial blood are represented as two buffer lines. The buffer line of the welling point blood is given as the mean slope ($-1.96 \log P$

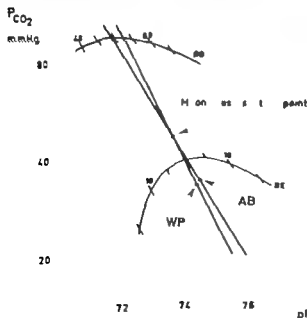


Fig. 6. The mean buffer lines of the arterial blood (AB) and the welling point (WP) blood. The mean Pco₂ obtained for AB in group I + II and the mean Pco₂ for WP in group I are indicated, as well as the mean pH of intersection of the buffer lines. For abbreviations and re-drawing of the diagram, see Fig. 5.

units) and the mean of the point of intersection between the two blood buffer lines from any single experiment ($P_{CO_2}/pH = 48.4/7.357$). The mean P_{CO_2} (± 1 S.D.) of the arterial and of the two groups was 34.2 ± 1.1 mmHg and that of the welling point blood 33.5 ± 1.2 mmHg (P_{CO_2} microelectrode recordings). The pH of the welling point blood obtained from osmogram (Fig. 6) was 7.437, thus slightly lower than the value of 7.448 of the arterial blood.

$$pH = pK + \log[HCO_3^-] - \log S \cdot P_{CO_2} \quad (1)$$

Actual bicarbonate concentration was calculated by using the Henderson-Hasselbalch equation (1), where pK is the apparent dissociation constant (6.10) for blood and S is the solubility coefficient of carbon dioxide (0.03 mM/mmHg) for blood (Siggaard Andersen 1974). For the arterial and welling point blood the $[HCO_3^-]$ was 22.9 and 21.7 mM respectively.

Discussion

P_{CO_2} microelectrode The silicone plug in the tip of the electrode must adhere properly to the surface of the tip so as to avoid leakage of water and electrolytes. Further the plug must be fairly thin, otherwise the response time will be too long. The lifetime of the electrode is influenced by the bicarbonate sensitivity of the antimony electrode. This phenomenon has been discussed by Karlmark and Sothell (1973) and Paschett and Zurbach (1974). This interference, however, does not affect the sensitivity or the validity of the electrode, as it is readily calibrated for known CO_2 tensions.

The empirical temperature coefficient for the antimony electrode (Vieira and Mainic 1968) is -2 mV/ $^{\circ}C$, which means about $0.05 \log P_{CO_2}/^{\circ}C$. This figure means an error of about 5 mmHg in the area 40 mmHg P_{CO_2} for each $^{\circ}C$. The influence of temperature is thus substantial. Checking of the temperature of the tissue surface, as well as of the bottom of the calibration cup, is therefore of great importance. This was done repeatedly by a thermistor with a water of 10 mm.

Intrarenal P_{CO_2} The intratubular P_{CO_2} range obtained in this investigation (40–85 mmHg) is in accordance with that reported by Karlmark and Danielson (1974) with use of equilibration technique. The values for intratubular P_{CO_2} obtained by direct measurements, supported the view that the P_{CO_2} of proximal tubular fluid is higher than that in the renal blood.

The high intraluminal P_{CO_2} was suggested by Brodsky and Schlicht (1974) to be due to the cellular production of CO_2 . However, this suggestion is contradicted by the finding of Swartz *et al.* (1971–1974) that the P_{CO_2} of the epithelial cells of the turtle bladder in the presence of exogenous carbon dioxide was about 2 mmHg, and by the fact that Mainic and Paschett (1976) calculated the intracellular P_{CO_2} to be, at best, 2–6 mmHg above that of the renal blood.

The neutralization reaction between filtered HCO_3^- and secreted H^+ was proposed by Swartz (1977) to be located at the border of the tubular lumen and cell, and it is therefore suggested that the high luminal P_{CO_2} probably emanates from this reaction. It is also sug-

gested that the reaction in itself should act as a limiting factor for CO_2 diffusion out of lumen. The P_{CO_2} of the luminal fluid has to be built up to the same level as that of reaction centre. Not until this level is reached will there be a gradient for CO_2 transport out of the lumen.

The chemical equilibrium—non-equilibrium situation in the proximal tubule Rector & (1965) and Vieira and Malnic (1968) found that the *in situ* pH of the tubular fluid is lower than the pH of a sample taken from the same tubular site and equilibrated to a P_{CO_2} of 40 mmHg. The authors proposed that the difference between these two pH was due to chemical non-equilibrium of the carbonic acid-bicarbonate system. The high P_{CO_2} in the proximal tubule found in the present study shows that the collected sample should not be equilibrated with a P_{CO_2} of the blood level or a P_{CO_2} of 40 mmHg but with that of the tubular fluid. The finding of a high intratubular P_{CO_2} thus seems to indicate that a chemical equilibrium exists between hydrogen ions, bicarbonate ions and carbon dioxide in the proximal tubular fluid. It is therefore probable that the Henderson-Hasselbalch equation (1) is valid in calculating one of the variables if the other two are known.

Welling point versus arterial blood The difference between the mean P_{CO_2} of the arterial blood in groups I and II combined and that of the welling point blood was 0.9 mmHg which is not statistically different from zero. It was shown, however, in the paired measurements in group I that the P_{CO_2} of the welling point blood was slightly lower than that of arterial blood ($P < 0.01$).

In the ultrafiltration process the efferent arteriolar blood will have a higher concentration of hemoglobin and plasma proteins as compared with the arterial blood. The buffer capacity will be increased due to the filtration process, as is shown by the higher absolute value of $\log P_{\text{CO}_2}/\text{pH}$ for welling point than for arterial blood. This is also illustrated by the fact that the increase in buffer base (BB) amounted to about 3 mM (Fig. 6) while the bicarbonate concentration was calculated to have decreased by 1.2 mM. The small increase in buffer capacity might explain the small change in pH and the small but statistically significant decrease of P_{CO_2} .

The rising protein concentration along the glomerular capillaries also means an increasing importance of the Donnan phenomenon, which thus slightly increases the local bicarbonate to the proximal tubules.

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References

- KEY W. A. and T. P. SCHLES, The masses of distinguishing between hydrogen secretion and bicarbonate secretion. Theory and application to the reptilian bladder and mammalian kidney. In: *Current topics membranes and transport*. New York: Academic, 1974, vol. 3, 161-224.
- MARK, B. and B. G. DANFORTH, Tricarbale acid, PCO₂, bicarbonate and ammonium ions along the rat renal tubule. *Acta physiol. scand.* 1974, 91, 243-258.
- MARK, B. and M. SÖRTTELL, The determination of bicarbonates in nanoliter samples. *Anal. J. Biochem.* 73, 53-1-11.
- MARK, B., M. SÖRTTELL and H. R. ULFENDAM, A pH-glass electrode for nanoliter biological samples. *Scand. Med. Scand.* 1971, 76, 58-62.
- SCOTT, G. and G. GILBERT, Cellular aspects of renal tubular acidification. *Physiol. Rev.* 1976, 56, 511-524.
- SÖRTTELL, J. B. and P. E. ZIMMACK, Re-evaluation of microelectrode methodology for the *in vitro* determination of pH and bicarbonate concentration. *Kidney Int.* 1974, 6, 81-91.
- SCOTT, F. C., N. W. CARTER and D. W. SELLERS, with the technical assistance of A. C. NUTT, The secretion of bicarbonate reabsorption in the proximal and distal tubules of the kidney. *J. clin. Invest.* 1965, 44, 278-290.
- WARTZ, J. H., J. T. FOWK, G. V. DAVIS and P. R. STEINMETZ, Distribution of metabolic CO₂ and the reported ionic species in acidification by turtle bladder. *Amer. J. Physiol.* 1974, 226, 283-289.
- WARTZ, J. H. and P. R. STEINMETZ, CO₂ requirements for H⁺ secretion by the isolated turtle bladder. *Am. J. Physiol.* 1971, 220, 2051-2057.
- WONKAL, L. W. and A. F. BRADLEY, Electrodes for blood PO₂ and PCO₂ determination. *J. appl. Physiol.* 8, 11, 515.
- WONKAL, L. W., *The acid-base status of the blood*. Munksgaard, Copenhagen, 3rd Ed. 1967.
- WONKAL, L. W., A phenomenological evaluation of CO₂-diffusion restriction in the kidney tubules studied in an artificial membrane system. *Acta physiol. scand.* 1979, 103, 129-136.
- WONKAL, L. W. and B. KARLMARK, *In vivo* microelectrode PCO₂ measurements. *Pflügers Arch. ges. Physiol.* 1976, 361, 179-180.
- WONKAL, L. W. and G. MALM, Hydrogen ion secretion by rat renal cortical tubules as studied by an antimony microelectrode. *Amer. J. Physiol.* 1968, 214, 710-718.

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The chemical equilibrium—non-equilibrium situation in the proximal tubule Rectora (1965) and Vieira and Malnic (1968) found that the in situ pH of the tubular fluid is lower than the pH of a sample taken from the same tubular site and equilibrated to P_{CO_2} of 40 mmHg. The authors proposed that the difference between these two pH was due to chemical non-equilibrium of the carbonic acid-bicarbonate system. The high P_{CO_2} in the proximal tubule found in the present study shows that the collected sample should not be equilibrated with a P_{CO_2} of the blood level or a P_{CO_2} of 40 mmHg but with that of the luminal fluid. The finding of a high intratubular P_{CO_2} thus seems to indicate that a chemical equilibrium exists between hydrogen ions, bicarbonate ions and carbon dioxide in the peritubular fluid. It is therefore probable that the Henderson-Hasselbalch equation (1) is of use in calculating one of the variables if the other two are known.

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alone medium (pH 7) carbon dioxide rather would react with hydroxyl ions and the H^+ appears to have a site of OH⁻ or HCO₃⁻ and not for CO₂ or H₂CO₃ (Maren 1967). The high entirely intracellular pH is, however, improbable, but could very well exist on the brush border surface (Craxford et al. 1938). This means that the OH⁻ ion, which is available by redox splitting of water leaves the H⁺ ion for accretion and reacts with carbon dioxide at the enzyme.

The neutralization reaction between H⁺ and HCO₃⁻ ions and the dehydration of H₂CO₃ has been extensively investigated with micropuncture techniques since it has been possible to obtain pH measurements in the tubules in vivo. With glass microelectrodes Rector (1965) compared the in situ pH with the pH measured in a sample taken from the same tubule and equilibrated to a P_{CO₂} of 40 mmHg. Under control conditions they found no difference in this respect in the proximal tubules. After inhibiting the carbonic anhydrase with acetazolamide, the in situ pH was 0.85 pH units lower than the pH in the equilibrated sample. This difference was thought to be due to the inhibition of the catalyzed dehydration of carbonic acid in the tubular lumen. This would lead to an accumulation of the carbonic acid and thus also of free hydrogen ions. The authors concluded that an enzyme localized on the tubular luminal membrane would prevent this accumulation. By inhibiting carbonic anhydrase with acetazolamide and using antimony pH microelectrodes Vieira and Malnic (1966) basically confirmed these results. Their pH difference was only 0.41 pH units, however. Both groups considered the pH difference to be due to a chemical nonequilibrium.

Karlmark and Danneberg (1974) measured the proximal intratubular pH in rats under control conditions and combined the results with the buffer lines obtained with a CO₂ titration method (Karlmark and Sottele 1973). Assuming a luminal chemical equilibrium they were then able to calculate the intratubular P_{CO₂}. The urine P_{CO₂} was found to be higher than the P_{CO₂} in the arterial blood. Recently Sottele and Karlmark (1976) and Sottele (1978 b) measured the intratubular P_{CO₂} with a microelectrode and found that it differs from the blood P_{CO₂} and that an intraluminal chemical equilibrium exists.

The aim of this investigation was to determine the pH bicarbonate concentration and the partial pressure of CO₂ along the rat proximal tubule under control conditions and during administration of acetazolamide in order to cast light on the problems concerning the equilibrium nonequilibrium situation for pH and P_{CO₂} in the proximal tubular lumen.

Material and Methods

The rats (Sprague-Dawley strain, Astarma, Stockholm, Sweden) weighing 205–285 g, fed on a diet of standard rat pellets (Astarma, Sweden) were used. They were allowed food and water ad libitum until the start of the experiment. The rats were anesthetized by an intraperitoneal injection of Inactin (Ciba, Fribourg, GmbH, Habsburg, W. Germany) in a dose of 120 mg/kg body weight (b.w.).

The body temperature was kept at 37.5°C with a servo-controlled heating table. The rats were tracheotomized and a catheter was inserted into the left femoral vein for infusions. The left femoral artery was cannulated for blood sampling and the right femoral artery for blood pressure measurements. The urinary bladder was drained to avoid diminished urine flow (Karlmark and Danneberg 1974). The left kidney was exposed through a flank incision and immobilized in a lucite cup by embedding it in 2% agar-agar in Ringer's solution. The exposed dorsal surface was superfused with warmed paraffin oil, preoxygenated with 5% CO₂ in distilled and 20% oxygen in nitrogen. The left ureter was catheterized.

For the TF P studies an incision was made, with FITC-antibodies as test substance. A continuous infusion of

CO₂ along the proximal tubules in the rat kidney

By

MORGAN SONTELL

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Abstract

SONTELL, M. CO₂ along the proximal tubules in the rat kidney. *Acta physiol. scand.* 105: 146-155.

The proximal intratubular pH of the rat kidney was measured in vivo with an antimony electrode. Pco₂ and bicarbonate concentration of the proximal tubular fluid were determined in an open equilibration system. The tubular fluid to plasma fluid concentration ratio was evaluated by a new fluorometric method. The acid-base parameters and the fluid concentrations were determined under control conditions and during acetazolamide treatment. The intratubular Pco₂ was higher than the systemic arterial blood under control conditions and the difference in Pco₂ was increased during acetazolamide treatment. In acetazolamide treated rats the rate of fractional bicarbonate reabsorption decreased in the early part of the proximal tubule while it was of about the same in the middle and late part as compared with control rats. The total bicarbonate reabsorption in the proximal tubule was about 50% due to the carbonic anhydrase inhibition. It seems possible that the bicarbonate is still reabsorbed after CO₂ after carbonic anhydrase inhibition, as hydrogen ion secretion is not totally stopped by this treatment. The increase in intratubular Pco₂ after acetazolamide treatment is assumed to be due to an inhibition of carbonic anhydrase facilitating effect on outward diffusion of CO₂ from the tubular lumen across the wall.

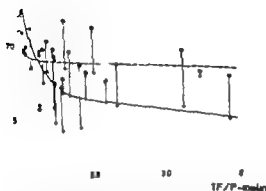
The urine in mammals may become acid either by reabsorbing filtered buffer anions, e.g. HCO₃⁻ and HPO₄²⁻, or by the addition of acid from the tubular cells to the fluid. It is clear from the findings of Pitts and Alexander (1945) that a tubular hydrogen ion secretion had to exist. The secreted hydrogen ion will then react with the filtered bicarbonate forming carbonic acid which is decomposed into water and carbon dioxide. Rector (1969) and Malnic and Steinmetz (1976) and Malnic and Giebisch (1976) have proposed that the portion of the bicarbonate reabsorption is mediated by the hydrogen-ion secretion. Rector (1969) however has suggested a direct HCO₃⁻ reabsorbing mechanism which in the proximal tubule should be responsible for about 60% of the total reabsorption.

One intracellular source of the secreted hydrogen ions is generally considered to be carbonic acid formed by the hydration of carbon dioxide. This reaction is catalyzed by carbonic anhydrase, which enzyme is present in the rat tubular cells, both in the cytoplasm and in the cell membranes (Lönnerholm 1971 and Wistrand and Kinne 1977). The hydrogen ions might also emanate from the splitting of water into hydrogen and hydroxyl ions.

pH

75

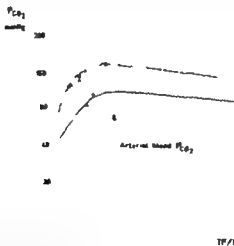
Arterial blood pH



The intracellular pH under control conditions (filled circles and solid line) and during acetazolamide (open circles and dashed line), at the same pressure was, plotted against the fractional length of the vessel under control conditions. The lines are drawn by eye. The dotted line indicates the mean arterial

Results

mean blood acid-base parameters did not differ significantly between the control period and period of acetazolamide treatment, according to paired *t*-test evaluation. Thus the values (± 1 S.D.) of pH, HCO₃⁻, P_{CO₂}, and base excess for the control periods were 7.41 ± 0.02 , 23.7 ± 1.8 mM, 37.8 ± 3.4 mmHg and -0.3 ± 1.8 mM and during acetazolamide 7.41 ± 0.03 , 24.0 ± 1.6 mM, 38.7 ± 3.9 mmHg and -0.1 ± 1.6 mM respectively.



The intracellular P_{CO₂} under control conditions (filled circles and solid line) and during acetazolamide (open circles and dashed line), at the same pressure was, plotted against TF/P-ratio under control conditions. The lines are drawn to the best visual fit. The dotted line indicates the mean arterial blood P_{CO₂}.

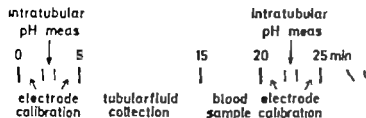


Fig. 1 The experimental arrangement.

30 ml/kg b.w. and hour of 40 mg/ml FITC-inulin in Ringer solution was given. The inulin (Kabi Spånga, Sweden) was labelled with fluorescein isothiocyanate (Pharmacia Fine Chemicals, Uppsala, Sweden). After 1 h the infusion gave a steady plasma concentration of about 1 mg/ml. In experiments the carbonic anhydrase activity was inhibited the FITC-inulin Ringer solution was replaced by 2 acetazolamide (Diamox® Lederle, Pearl River NY), and 40 mg/ml FITC-inulin in 130 mM L-bicarbonate solution. The sodium bicarbonate was added to keep the acid-base status of the rats the two situations studied. The infusion was continued for 30 min before any sample was taken. The mental arrangement is shown in Fig. 1.

Intratubular pH The intratubular pH was measured with an antimony electrode system (Karlmark designed for micropuncture, in a manner similar to that described by Vieira and Maltbie (1962)). The electrodes were ground to a diameter of about 5 μ m. The silver-silver chloride reference electrode had a junction of 1 M KCl (2 μ agar) in a glass cannula with a tip of 7 μ m. The tip was inserted into the parenchyma and the location was not critical for the pH measurements. The electrodes are connected to a mv meter (PHM 26, Radiometer Copenhagen, Denmark). They were calibrated before and the intratubular measurement, using phosphate buffer solutions placed in a holder adjacent to the lumen and thermostated to the kidney temperature.

The sensitivity of the electrode system was of the same order as that described by Karlmark (1971) Karlmark and Sohtell (1973). It was found necessary, however, to re-investigate the bicarbonate interference described by Karlmark and Sohtell (1973) because of the variation of the antimony quality and the electrode grinding. The interference was measured as described in the paper cited and was found to be pH units lower than the true pH value. All pH measurements containing bicarbonate recorded with antimony electrode have therefore been corrected. Proximal tubules were identified as the non-S₁ segments and were randomly punctured.

Intratubular sampling After the pH measurements, tubular fluid was sampled at a rate of 3-4 μ l using a collection method described by Karlmark and Danielson (1974). The puncture site was the same as the pH measurements and the tubular urine sampling. During the period of acetazolamide infusion, the puncture site was re-investigated for pH recording and sample collection. The results from the two sites could thus be compared at the same anatomical site. This point is expressed as TF/P inulin under the conditions.

Inulin determination. A fluorescence method described by Rutih *et al.* (1976) was modified and we measure the FITC-inulin concentrations in plasma and urine (Sohtell *et al.* 1978). Duplicate samples of 20 μ l each of tubular fluid and arterial blood plasma were assayed for their FITC-inulin concentrations. The TF/P ratio of the FITC-inulin indicated the distance between the glomerulus and the punctured site. In mind that TF/P inulin does not linearly represent the length.

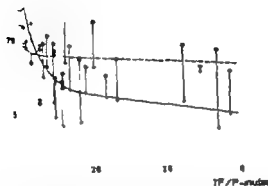
Intratubular P_{CO_2} and bicarbonate determinations. The intratubular fluid samples were equilibrated to different P_{CO_2} and the pH was measured at each gaseous tension by means of a glass microelectrode (Lund *et al.* 1971). From the measured intratubular pH and the pH/log P_{CO_2} line, the intratubular P_{CO_2} was determined assuming conditions of equilibrium. The bicarbonate concentration of the sample and particular pH and P_{CO_2} was then calculated using the Henderson-Hasselbalch equation. Aspects of determinations have been discussed by Karlmark and Sohtell (1973) and Sohtell and Karlmark (1975).

Acid-base of the blood. The acid-base status of the rat was determined as that of the arterial blood according to the method of Siggaard Andersen (1967). The bicarbonate concentration was evaluated for plasma water supposing the volume of plasma proteins is 6% of plasma volume (Kunin 1972). For the 400 μ l of blood was drawn from the left femoral artery into heparinized syringe without section. This volume was taken for determination of the plasma FITC-inulin concentration. After transient of the blood pressure should not be below 100 mmHg, otherwise the experiment was ended.

pH

7.5

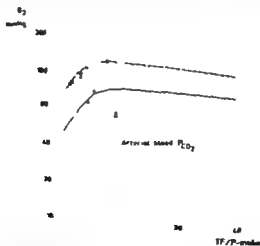
Arterial blood pH



The intratubular pH under control conditions (filled circles and solid line) and during acetazolamide (open circles and dashed line), at the same puncture site, plotted against the functional length of tubule under control conditions. The lines are drawn by eye. The dotted line indicates the mean arterial

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The intratubular P_{CO₂} under control conditions (filled circles and solid line) and during acetazolamide (open circles and dashed line), at the same puncture site, plotted against TF/P-uride under control conditions. The lines are drawn to the best visual fit. The dotted line indicates the mean arterial blood P_{CO₂}.

TABLE I Results from 47 proximal tubules from 10 rats. C=control conditions and I=inhibition of carbonic anhydrase activity

Tubule	Intra-tub pH	Intra-tub. P_{CO_2} mmHg	Intra-tub. HCO_3^- mM	Intra-tub. pH at blood P_{CO_2}	Non-equil. pH	TF/P Inulin	Blood pH	Blood P_{CO_2} mmHg	Plasma HCO_3^- mM	In H^+ For H^+
1c	6.84	36.0	6.1	6.82	+0.0	1.61	7.43	37.5	4.7	+11
1i	7.16	53.0	18.7	7.34	-0.18	1.4	7.39	34.3	20.5	1.
2c	6.39	115	6.9	7.39	-0.55	3.72	7.38	34.8	20.5	0
3c	6.44	—	—	—	—	1.81	7.38	34.8	20.5	-11
4c	6.83	65.0	6.0	6.84	-0.26	1.24	7.38	34.8	20.5	0
2i	6.91	125	24.8	7.36	-0.45	1.79	7.35	42.5	23.0	1.
3	6.86	121	21.4	7.31	-0.45	1.40	7.35	42.5	23.0	1.
4i	6.57	243	22.0	7.28	-0.71	1.22	7.35	42.5	23.0	1.
5c	6.61	128	12.7	7.07	-0.46	2.18	7.40	40.9	25.0	11
6c	6.42	159	10.2	6.94	-0.52	1.55	7.40	40.9	25.0	+11
7c	6.59	80.0	7.6	6.85	-0.26	2.33	7.40	40.9	25.0	+11
5i	6.75	202	27.7	7.39	-0.64	1.90	7.40	38.0	23.2	-11
6i	6.71	227	28.4	7.39	-0.68	1.22	7.40	38.0	23.2	11
7i	6.86	148	26.1	7.36	-0.50	1.63	7.40	38.0	23.2	11
8c	6.48	132	9.7	6.87	-0.39	1.45	7.39	44.4	26.0	+11
9c	6.67	82.5	9.4	6.90	-0.23	1.57	7.39	44.4	26.0	11
10c	6.64	90.0	9.6	6.88	-0.24	1.89	7.39	44.4	26.0	11
8i	6.74	197	26.4	7.18	-0.54	1.34	7.37	46.0	25.6	+11
9i	6.77	186	26.7	7.31	-0.54	1.25	7.37	46.0	25.6	11
10i	6.81	176	27.7	7.32	-0.51	1.50	7.37	46.0	25.6	+11
11c	6.77	87.0	12.5	7.15	-0.38	3.49	7.43	36.5	23.9	11
12c	6.57	85.0	7.7	6.94	-0.37	3.26	7.43	36.5	23.9	+11
11i	6.79	164	24.6	7.48	-0.69	1.00	7.44	36.8	24.7	1.
12i	6.94	137	29.1	7.48	-0.54	—	7.44	36.8	24.7	+1.
13c	6.49	101	7.6	6.92	-0.43	3.90	7.40	38.2	23.3	-11
14c	6.66	69.0	7.7	6.92	-0.26	1.67	7.40	38.2	23.3	-11
15c	6.64	100	10.6	7.07	-0.43	1.67	7.40	38.2	23.3	11
13i	6.77	179	25.7	7.44	-0.67	1.67	7.40	41.8	25.4	+11
14i	6.95	119	25.8	7.43	-0.48	—	7.40	41.8	25.4	+11
16c	6.99	69.0	16.4	7.26	-0.27	1.06	7.44	37.6	25.2	+11
17c	6.75	72.0	9.9	7.02	-0.27	1.30	7.44	37.6	25.2	+11
16i	6.94	112	23.8	7.35	-0.41	1.06	7.4	40.2	25.8	+1
17i	6.97	119	27.1	7.41	-0.44	1.15	7.42	40.2	25.8	+1
18c	6.58	56.0	5.2	6.78	-0.20	1.45	7.44	31.6	21.5	1
19c	6.81	68.5	10.8	7.10	-0.29	2.01	7.44	31.6	21.5	1
20c	6.96	37.5	8.3	7.02	-0.06	1.26	7.44	31.6	21.5	+1
18	6.97	86.0	19.6	7.34	-0.37	1.13	7.45	35.0	24.5	1
19i	7.11	64.0	20.1	7.34	-0.23	1.16	7.45	35.0	24.5	+1
20i	6.93	115	23.9	7.40	-0.47	1.13	7.45	35.0	24.5	+1
21c	7.09	63.5	19.0	7.31	-0.22	0.96	7.39	37.8	22.5	1
21i	6.96	107	23.8	7.54	-0.58	1.09	7.45	34.0	23.5	+1
22c	6.82	67.5	10.9	7.06	-0.24	1.35	7.42	38.2	24.5	+1
23c	6.90	49.0	9.5	7.01	-0.11	1.45	7.42	38.2	24.5	+1
24c	7.11	57.0	17.9	7.28	-0.17	1.13	7.42	38.2	24.5	+1
22i	7.02	111	28.3	7.47	-0.45	1.13	7.40	38.7	23.6	1
23i	6.92	139	28.2	7.44	-0.5.	1.22	7.40	38.7	23.6	1
24i	6.84	168	28.3	7.45	-0.61	1.08	7.40	38.7	23.6	1

$$TF/P_{HCO_3} = 2.8$$

The tubular fluid-plasma ratio of bicarbonate concentration (filled circles) and during acetazolamide (open circles), in relation to TF/P-inulin under control



tubular pH. As discussed in a previous paper (Sobteff 1977) a touch on the luminal membrane with a measuring electrode would show a potential recording giving a false value. By moving the electrode from the cell border such a potential effect was avoided. Fig. 2 the intratubular pH measured during the control and acetazolamide periods is shown, in relation to the functional length of the tubule under control conditions (TF/P-inulin).

Intracellular P_{CO_2} values were obtained from the pH/log P_{CO_2} line and the corresponding tubular pH, as previously described. In Fig. 3 the intratubular P_{CO_2} under control and acetazolamide conditions is shown in relation to TF/P-inulin in the control situation. It is seen that the P_{CO_2} increased rapidly in the early parts of the proximal tubule, with a tendency to decline in the later parts. Most values were higher than the P_{CO_2} of the arterial blood (0.001). Further the P_{CO_2} values obtained during acetazolamide administration were lower than those in the control situation ($P < 0.001$).

Intracellular bicarbonate concentration. In the calculations of bicarbonate the constants K_1 in the Henderson-Hasselbalch equation were $pK_a = 6.101$ and $S = 0.0307$ which are valid for the ultrafiltrate of the rat plasma (Sobteff and Karlmark 1978). These values are, moreover, in agreement with those of human plasma filtrate (Steja 1962, Mitchell *et al.* 1965 and Sobteff 1971) and with those calculated as described by Hastings and Sordroy (1959) and Van Slyke *et al.* (1928). The bicarbonate concentrations are seen in Table I.

In Fig. 4 the tubular fluid-plasma ratio (TF/P) for bicarbonate under control and acetazolamide conditions is shown as a function of TF/P-inulin in the control situation.

Discussion

Karlmark and Sobteff (1973), Malnic *et al.* (1974) and Puscheck and Zurbach (1974) have reported that falsely low pH values are recorded with the antimony electrode when it is used for measurements in solutions containing bicarbonate and is standardized in phosphate buffer. The differences ranged between 0.07 and 0.18 pH units. If the properties of the electrodes are changed, and with somewhat different standardizations, however a re-evaluation of the magnitude of the error is required for every experimental set-up. In this investiga-

TABLE I. Results from 47 proximal tubules from 10 rats. C—control conditions and I—infusion of anhydrous activity

Tubule	Intra- tub pH	Intra- tub Pco ₂ mmHg	Intra- tub. HCO ₃ ⁻ mM	Intra- tub. pH at blood Pco ₂	Non- equil. pH	TF/P inulin	Blood pH	Blood Pco ₂ mmHg	Plasma HCO ₃ ⁻ mM	La mEq/L
1c	6.84	36.0	6.1	6.82	+0.02	1.61	7.43	37.5	24.7	0
1i	7.16	53.0	18.7	7.34	-0.18	1.42	7.39	34.3	20.5	1
2c	6.39	115	6.9	7.39	-0.55	3.72	7.38	34.8	20.5	0
3c	6.44	—	—	—	—	1.81	7.38	34.8	20.5	0
4c	6.83	65.0	6.0	6.84	-0.26	1.24	7.38	34.8	20.5	0
2i	6.91	123	24.8	7.36	-0.45	1.79	7.35	42.5	23.0	0
3i	6.86	121	21.4	7.31	-0.43	1.40	7.35	42.5	23.0	0
4i	6.57	43	2.0	7.28	-0.71	1.22	7.35	42.5	23.0	0
5c	6.61	128	12.7	7.07	-0.46	2.18	7.40	40.9	25.0	+0
6c	6.42	159	10.2	6.94	-0.5	1.55	7.40	40.9	25.0	+0
7c	6.59	80.0	7.6	6.83	-0.26	2.33	7.40	40.9	25.0	+0
5i	6.75	202	27.7	7.39	-0.64	1.50	7.40	38.0	23.2	+0
6i	6.71	227	28.4	7.39	-0.68	1.24	7.40	38.0	23.2	+0
7i	6.86	148	76.1	7.36	-0.50	1.63	7.40	38.0	23.2	+0
8c	6.48	132	9.7	6.87	-0.39	1.45	7.39	44.4	26.0	+0
9c	6.67	82.5	9.4	6.90	-0.23	1.57	7.39	44.4	26.0	0
10c	6.64	90.0	9.6	6.88	-0.24	1.89	7.39	44.4	26.0	0
8i	6.74	197	26.4	7.28	-0.54	1.34	7.37	46.0	25.6	+0
9i	6.77	186	26.7	7.31	-0.54	1.25	7.37	46.0	25.6	+0
10i	6.81	176	27.7	7.3	-0.51	1.50	7.37	46.0	25.6	0
11c	6.77	87.0	10.5	7.15	-0.38	3.49	7.43	36.5	23.9	0
12c	6.57	85.0	7.7	6.94	-0.37	3.26	7.43	36.5	23.9	+0
11i	6.79	164	24.6	7.48	-0.69	1.00	7.44	36.8	24.7	0
12i	6.94	137	29.1	7.48	-0.54	—	7.44	36.8	24.7	0
13c	6.49	101	7.6	6.92	-0.43	3.90	7.40	38.2	23.3	0
14c	6.66	69.0	7.7	6.9	-0.6	1.67	7.40	38.2	23.3	+0
15c	6.64	100	10.6	7.07	-0.43	1.67	7.40	38.2	23.3	+0
13i	6.77	179	25.7	7.44	-0.67	1.67	7.40	41.8	25.4	+0
14i	6.95	119	35.8	7.43	-0.48	—	7.40	41.8	25.4	+0
16c	6.99	69.0	16.4	7.26	-0.27	1.06	7.44	37.6	25.2	+0
17c	6.75	72.0	9.9	7.02	-0.27	1.30	7.44	37.6	25	+0
16i	6.94	112	35.8	7.35	-0.41	1.06	7.4	40.2	25.8	+0
17i	6.97	119	27.1	7.41	-0.44	1.15	7.42	40.2	25.8	+0
18c	6.58	56.0	5.2	6.78	-0.20	1.45	7.44	31.6	1.5	0
19c	6.81	68.5	10.8	7.10	-0.29	2.01	7.44	31.6	1.5	0
20c	6.96	37.5	8.3	7.02	-0.06	1.26	7.44	31.6	21.5	0
18i	6.97	86.0	19.6	7.34	-0.37	1.13	7.45	35.0	4.5	+0
19i	7.11	64.0	20.1	7.34	-0.23	1.26	7.45	35.0	4.5	+0
20i	6.93	115	23.9	7.40	-0.47	1.13	7.45	35.0	4.5	+0
21c	7.09	63.5	19.0	7.31	-0.22	0.96	7.39	37.8	22.5	0
1i	6.96	107	23.8	7.54	-0.58	1.09	7.45	34.0	23.5	+0
22c	6.82	67.5	10.9	7.06	-0.24	1.35	7.42	38.2	24.5	+0
23c	6.90	49.0	9.5	7.01	-0.11	1.45	7.42	38	24.5	+0
24c	7.11	57.0	17.9	7.28	-0.17	1.13	7.42	38.2	4.5	+0
22i	7.02	111	28.3	7.47	-0.45	1.13	7.40	38.7	23.6	0
23i	6.92	139	28.2	7.44	-0.5	1.22	7.40	38.7	23.6	0
4i	6.84	168	78.3	7.45	-0.61	1.08	7.40	38.7	23.6	0

barious, as pH and P_{CO_2} are not constant along the tubules, which means that the tions are greatly dependant on the puncture site from which the recordings and samples ate.

sky and Schöb (1974) suggested that an elevation of the P_{CO_2} of the lumen might e to an intracellular carbon dioxide production. Malnic and Giebisch (1976) have, ver calculated the intracellular P_{CO_2} to be, at best, 2-6 mmHg above that of the venous 1 and/or the tubular fluid. Schwartz *et al.* (1971-1974) have also calculated the P_{CO_2} lthelial cells of the turtle bladder to be about 2 mmHg in the absence of exogenous n dioxide. It is therefore unlikely that the high intratubular P_{CO_2} reflects a high intra lter P_{CO_2} .

w investigations have been made concerning the carbon dioxide permeation across gical membranes. Constantino *et al.* (1962) measured the half time for carbon dioxide ke by erythrocytes and found it to be about 0.1 second, and the same value was found rew *et al.* (1963) in similar experiments.

on the chemical reaction



and Marten (1972) determined the rate constants K_1 and K_2 at 37°C and found a half of 0.014 second for K_1 (uncatalyzed). This reaction half time indicated that the trans- of CO through erythrocyte membranes rather is restricted in CO diffusion than de lent on the uncatalyzed dehydration reaction.

experiments designed to evaluate the acidification kinetics in single nephron segments, he *et al.* (1974) provided some evidence of restriction of CO diffusion through the ural tubular wall. A half time of 0.5 to 1.0 s was found for the carbon dioxide equ- tion in air-equilibrated solution perfusing the proximal tubules, and this was much er than the half time of getting an equilibrium in the CO hydration- H_2CO_3 dehydration ion, even without carbonic anhydrase. They suggested that the equilibration rate is ed by the diffusion of carbon dioxide across the tubular wall.

ohell (1978 b) demonstrated by direct intratubular measurements that the P_{CO_2} in the ximal tubules varied between 40 and 85 mmHg in rats under control conditions. This ity strengthens the result of the present investigation. The similarity of this range for control situation in the present study to that found by Karlmark and Danielson (1974) ngly supports the existence of chemical equilibrium in the reactions within the carbonic 3 system contained in the tubular fluid. This qualifies the equilibration method (Karl- rk and Soblert 1973) for use in calculating the intratubular P_{CO_2} . The high intratubular as compared with the blood P_{CO_2} points to a restriction of the CO diffusion out of lones.

It was proposed by Soblert (1978 a), from investigations of the neutralization reaction ecca H^+ and HCO_3^- ions in a multi-membrane model, that the reaction in itself causes variation of the CO diffusion if the reaction centre is located close to the membrane of l luminal side. This means that the luminal P_{CO_2} has to be increased to a level equal to the P_{CO_2} level in the reaction centre, if a net outward transport of carbon dioxide is to take place.

$$\frac{TF/P_{HCO_3}}{TF/P_{inulin}}$$

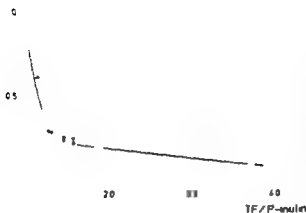


Fig. 5. The fractional bicarbonate excretion under control and during inhibition of carbonic anhydrase activity (open and filled circles and solid and dashed line), plotted as $TF/P_{HCO_3}/TF/P_{inulin}$ under control conditions. The lines are drawn

tion pH values obtained with the antimony electrode were corrected for an error of 0.05 units (see Material and Methods)

Direct measurements of the pH within the rat proximal tubules under free-flow or perfusion in normal situations and during carbonic anhydrase inhibition have been reported in the literature (see Rector 1976). The pH patterns along the proximal tubules (Fig. 2) in the present investigations, under control conditions are consistent with the results obtained earlier. A remarkable change of pH is seen when acetazolamide is given. In the early proximal tubule ($TF/P_{inulin} < 1.26$) the pH is lower than under control conditions, but in the later parts it is higher. Quehenberger *et al.* (1977) reported similar results, but in a transient pH decrease in the early proximal tubules just after benzolamide infusion. The functional background of the change of the pH pattern after administration of acetazolamide is not fully understood.

Under control conditions the bicarbonate reabsorption expressed as fractional bicarbonate excretion, in the early part of the proximal tubule was much faster than in the late part (Fig. 5). After the administration of acetazolamide the reabsorption was almost the same along the whole tubule and of about the same rate as in the late tubule under control conditions. These discrepancies under the two conditions might explain the results presented in Fig. 2. An altered pH pattern would also be the result of CO_2 diffusion across the tubular membrane was restricted and H^+ secretion into the tubular lumen decreased on administration of acetazolamide. If so the restricted CO_2 diffusion might be explained by the increased intratubular P_{CO_2} after acetazolamide treatment, as shown in Fig. 3.

The difference between the *in situ* pH and the "equilibrated sample" pH obtained in the control situation is identical with that calculated from the data of Karlmark and Danneberg (1974), namely 0.29 which is statistically different from zero ($P < 0.001$). Rector (1965) and Vieira and Malnic (1968) found no such difference, except during carbonic anhydrase inhibition. Rector *et al.* then found a value of 0.85 (benzolamide) and Vieira and Malnic 0.41 (acetazolamide), which may be compared with the 0.51 pH units found in this investigation (acetazolamide). Comparison of the mean pH-difference values is

11. G. and G. GERSHOCK, Cellular aspects of renal tubular acidification. *Physiologist* 1976. 19 511-524
12. G. and P. R. STEINMETZ, Transport processes in urinary acidification. *Kidney Int.* 1976. 9 172-188.
13. G. G., M. MILLO-AMIEL and A. C. CAMOLA, Kinetic analysis of renal tubular acidification by anti-*o*-microelectrodes. In: *Ion selective microelectrodes*. New York: Plenum, 1974. 89-108.
14. T. H., Carbonic anhydrase: Chemistry, physiology and inhibition. *Physiol. Rev.* 1967 47 595-781.
15. T. H., Renal carbonic anhydrase and the pharmacology of sulfonamide inhibitors. *Handb. Exp. med.* 1969 24 195-256
16. HILL, R. A. D. A. HILLBERT and C. T. CARMAN, Acid-base constants and temperature coefficients for brookland fluid. *J. appl. Physiol.* 1965. 20-1 27-30.
17. R. F. and R. S. ALEXANDER, The nature of the renal tubular mechanism for acidifying the urine. *or J. Physiol.* 1945 144 239-254.
18. HILL, J. B. and P. E. ZORRACH, Re-evaluation of microelectrode methodology for the *in vitro* determination of pH and bicarbonate concentration. *Kidney Int.* 1974 6 81-91
19. ENGBERG, P. F. LANG, R. GERSHOCK and P. DUSTON, Effect of bicarbonate on luminal pH under free flow. *Am. J. Physiol.* 1977 Vol. 368.
20. or, F. C. Renal acidification and endocrine production: chemistry of weak acids and bases; buffer capacity. In: *The Kidney* W. B. Saunders Publishers, 1976 318-342.
21. or, P. C. M. W. CARTER and W. W. SELBY, with the technical assistance of A. C. NICHOL, The mechanism of bicarbonate reabsorption in the proximal and distal tubules of the kidney. *J. clin. Invest.* 1965. 2 278-290.
22. or, H. C. D. W. SELBY, A. D. ROBERTS and J. E. SMITH, The role of plasma CO₂ tension and carbonic dehydratase activity in the renal reabsorption of bicarbonate. *J. clin. Invest.* 1960. 39 1709-1721
23. U. G. K. E. ALPHEI and H. R. ULSTED, Fluorescence measurements in microtitre samples. *Anal. Chem.* 1976. 72 539-545.
24. HILL, J. H. J. T. FINE, G. VADONI and P. R. STEINMETZ, Distribution of metabolic CO₂ and the transmembrane species in acidification by turtle bladder. *Amer. J. Physiol.* 1974. 226 283-289
25. HILL, J. H. and P. R. STEINMETZ, CO₂ requirements for H⁺ secretion by the isolated turtle bladder. *Am. J. Physiol.* 1971. 220. 2051-2057
26. B. K., The solubility of carbon dioxide in the cerebral cortical tissue of cats. With notes on the solubility of carbon dioxide in water 0.16 M NaCl and cerebrospinal fluid. *Acta physiol. scand.* 1962. 55 5-341
27. B. K., The bicarbonate, carbonic acid buffer system of the cerebral cortex of cats, as studied in tissue suspensions. II. The pK_a of carbonic acid at 37.5°C, and the relation between carbon dioxide and pH. *Acta physiol. scand.* 1962. 55 2 121-141
28. JAAK ANDERSEN, O. The acid-base status of the blood. Munksgaard, Copenhagen, 3rd Ed. 1967
29. HILL, M. Electrochemical forces for chloride transport in the proximal tubules of the rat kidney. *Acta physiol. scand.* 1978. 103 363-369
30. HILL, M. A phenomenologic evaluation of CO₂-diffusion restriction in the kidney tubules studied in an artificial system. *Acta physiol. scand.* 1979. 105 129-136.
31. HILL, M. P_{CO₂} of the proximal tubular fluid and the efferent arteriole blood in the rat kidney. *Acta physiol. scand.* 1979. 105 137-143
32. HILL, M. and B. KARLMARK, *In vitro* microperfused P_{CO₂} measurements. *Pflügers Arch. ges. Physiol.* 1978. 381 179-180
33. HILL, M. and B. KARLMARK, Aspects on bicarbonate determination in biological fluids. To be published 1979
34. HILL, M. A.-C. ECKHORN and M. SJÖGREN, FITC-iodine—a suitable test substance in renal physiology. To be published.
35. HILL, D. D. J. SANDOZ, A. B. HARRISON and J. M. NEIL, Studies of gas and electrolyte equilibria in blood. The solubility of carbon dioxide at 38°C in a salt solution, serum and blood cells. *J. Biol. Chem.* 1928. 78 3 765-799
36. HILL, M. and G. H. M. DOB, Renal excretory mechanisms. Mineral Metabolism, Academic Press, New York and London, 1960 1 237-336
37. HILL, F. L. and G. MALM, Hydrogen ion secretion by rat renal cortical tubules as studied by an antimony microelectrode. *Amer. J. Physiol.* 1968. 216 4 710-718
38. HILL, P. J. and R. KRIV, Carbonic anhydrase activity of isolated brush border and basolateral membranes of the renal tubular cells. *Pflügers Arch. ges. Physiol.* 1977. 370 121-126.

Berliner (1957) Walser and Mudge (1960) and Rector *et al.* (1960) suggested the existence of a lumenally active carbonic anhydrase. Rector *et al.* (1965) proposed that this enzyme increased the dehydration rate of H_2CO_3 , preventing the accumulation of carbonic acid in the tubular lumen and, thus, reducing the chemical non-equilibrium pH. However the work of Malnic *et al.* (1974) pointed to the carbon dioxide diffusion as being the rate-limiting step rather than the dehydration reaction. Maren (1967) questioned the necessity of a lumenal carbonic anhydrase for the dehydration of H_2CO_3 .

The membrane bound enzyme found by Wistrand and Kinne (1977) would rather tend to facilitate the diffusion of carbon dioxide from the tubular lumen. This conclusion is to be plausible, as it is shown in the present investigation that the intraluminal pH_i significantly increased when the carbonic anhydrase is inhibited with acetazolamide. This view is also favoured by results of Karlmark *et al.* (1977), who found no significant change in the in situ pH measured in perfused segments of the proximal tubules when using a carbonic anhydrase inhibitors acting only in the tubular lumen.

The results of this investigation indicate the existence of a high P_{CO_2} in the proximal tubules, which might explain the earlier assumption of a non-equilibrium pH. The phenomenon is thus instead a non-equilibrium P_{CO_2} .

References

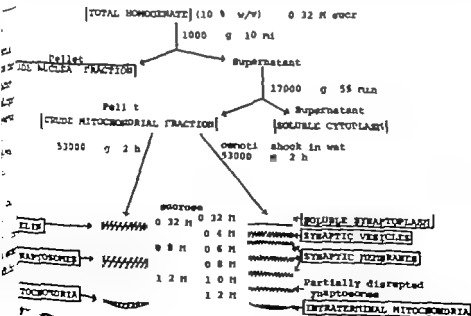
- BERLINER, R. W. Some aspects of ion exchange in electrolyte transport by the renal tubules. Microscopic aspects of transport across cell membranes. The University of Wisconsin Press. 1957. 203-220.
- BODANSKY, W. A. and T. P. SCHULZ. The means of distinguishing between hydrogen secretion and bicarbonate reabsorption. Theory and application to the reptilian bladder and mammalian kidney. In: *Current topics in membranes and transport*. New York. Academic. 1974. Vol. 6. 161-224.
- CONSTANTINE, H. P., M. R. CRAW, J. A. MORELLO and R. E. FOSTER. The rate of the reaction of CO_2 with dilute human red blood cell suspensions. *Physiology* 1962. 5. 120.
- CRAW, M. R., H. P. CONSTANTINE, J. A. MORELLO and R. E. FOSTER. Rate of the Bohr shift in human cell suspension. *J. appl. Physiol.* 1963. 18. 317-34.
- CRAWFORD, S. R., O. GATTY and T. TEORELL. A note on surface pH. *Philos. Mag.* 1932. 1061-1064.
- GARG, L. C. and T. H. MAREN. The rates of hydration of carbon dioxide and dehydration of carbonic acid at 37°C. *Biochim. Biophys. Acta (Amst.)* 1972. 261. 70-76.
- HASTINGS, A. B. and J. SENDROY. The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid. *Biol. Chem.* 1925. 65. 443-455.
- JOHNZELL, H. E. The first dissociation constant for carbonic acid and the solubility of carbon dioxide in man's amniotic fluid. *Scand. J. clin. Invest.* 1971. 27. 233-238.
- KARLMARK, B. The determination of titratable acid and ammonium ions in picomole amounts. *Acta Biochem.* 1973. 52. 69-82.
- KARLMARK, B. and B. O. DANIELSON. Titratable acid, P_{CO_2} , bicarbonate and ammonium ions along the proximal tubule. *Acta physiol. scand.* 1974. 112. 243-258.
- KARLMARK, B. and M. SONTELL. The determination of bicarbonate in nanoliter samples. *Anal. Biochem.* 1973. 53. 1-11.
- KARLMARK, B., B. ÅGERUP and P. J. WISTRAND. Carbonic anhydrase and renal tubular reabsorption of bicarbonate. Role of brush border and cytoplasmic enzyme. Submitted for publication.
- KARLMARK, B., M. SONTELL and H. R. ULFENDALE. A pH-glass electrode for nanoliter biological samples. *Acta Soc. Med. Scand.* 1971. 76. 58-62.
- KUMAI, J. and R. T. The influence of the carbonic anhydrase inhibitor, benzotamide (CL 11) on the reabsorption of chloride, sodium and bicarbonate in the proximal tubule of the rat. *J. clin. Invest.* 1971. 51. 294-306.
- LÖNNERDAL, O. Histochemical demonstration of carbonic anhydrase activity in the rat kidney. *Cell Tissue Res.* 1971. 81. 433-439.

Table 1. Properties of amino acids injected intracerebrally into mice in the present study

acid	Specific activity	Amount per animal	Radioactivity per animal	Biological nature of amino acid
serine	550 Ci/mol	56 nanomoles	20 μ Ci	Slowly-metabolizing, transmitter candidate
tryptophan	300 Ci/mol	2 nanomoles	250 nCi	Non-metabolizing, non-transmitter
tyrosine	18 Ci/nanomol	60 picomoles	1 μ Ci	Metabolizing, non-transmitter
isotonic acid	275 Ci/mol	1.25 nanomoles	300 nCi	Metabolizing, transmitter candidate

brain was opened by drifting hole 2 mm from the intersection of the sagittal and coronal sutures (Lyons and McCosack 1957; Noble *et al.* 1967). One μ l of the radioactive amino acids was injected through a 25-gauge syringe to a depth of 2 mm into the cortex. After removal of the needle the hole in the skull was covered with Fluon-55 resin. A total of 20 mice was treated similarly in each series of experiments, and the procedure was repeated four times with each amino acid. The properties and nature of the amino acids are in Table 1. All were obtained from the Radiochemical Centre, Amersham.

Mice were fasted and cataleptic for 4-5 h after the injection, but gradual normalization of their motor activities after 5 h was accompanied by progressive recovery of their normal alertness. The mice were then killed by decapitation 20 h after the injection, and the brains removed, pooled, weighed and rapidly homogenized in cold 0.32 M sucrose. The homogenates were fractionated according to the method of Whittaker and Barker (1972) and samples taken from each fraction for radioactivity and protein estimation (see Fig. 1). Protein was determined by the method of Lowry *et al.* (1951). For the measure-



Synaptosomal accumulation of intracerebrally injected amino

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Abstract

KARPPINEN, A., E. KUMPULAINEN and P. LÄHDESMÄKI. *Synaptosomal accumulation of intracerebrally injected amino acids*. Acta physiol. scand 1979 105 156-162.

The distribution of the labels of [35 S]taurine, [3 H]lysine, [14 C]glutamate and [14 C]norleucine in mouse brain subcellular fractions was followed after intracerebral injection. In the [35 S]taurine, [3 H]lysine and [14 C]glutamate and its metabolites accumulated in the nerve terminals, lysine and glutamate also occurring in synaptic vesicles, while taurine remained mainly in the soluble synaptoplasm. [14 C]norleucine passed the brain cell membranes slowly but was still bound to the synaptic vesicles to a greater extent than taurine.

Key words. Intracerebral injection, accumulation of amino acids, nerve terminals, synaptic organelles.

In agreement with the work of De Belleroche and Bradford (1973), we have observed the presence of 10 amino acids in calf brain synaptic vesicles (Lähdesmäki and Winter 1977, Lähdesmäki *et al.* 1977a), although some other workers assume that their observations on the occurrence of these same amino acids in the synaptic vesicle fraction are influenced by contamination from amino acids in the soluble synaptoplasm (Mangan and Whittaker 1966, Rassin 1972). One further approach to the study of this controversial question would be to follow the passage of injected amino acids into brain subcellular fractions (Rassin *et al.* 1977). This is complicated however by the effective blood brain barrier which prevents the passage of amino acids from the circulation into brain cells (Cohen and Lipton 1972). Thus intracerebral injection may provide a better opportunity for explaining the entrance of amino acids into brain cells. Farrow and O'Brien (1971) did in fact observe that intraventricularly injected [3 H]acetate and amino acids formed from it in synaptosomes and synaptic vesicles. The work reported here examines the distribution of the radioactivity of four different amino acids in mouse brain subcellular fractions in order to ascertain whether these are able to accumulate in nerve terminals and their subcellular organelles.

Material and methods

A total of 360 adult female white mice (NMRI) were used. They were anesthetized by an intramuscular injection of 200 μ l of 10% (w/v) chloral hydrate and immobilized on a small animal rack in the head horizontal position. A midsagittal incision was made from the eyes to the ears and the skull

II Recovery rates of the intracerebrally injected amino acids.

acid	Recovery %
taurine	21.4 ± 2.2
lysine	20.7 ± 1.8
glutamic acid	16.2 ± 2.0
norleucine	14.8 ± 2.4

percentage recovery rates are calculated from the counts obtained in the total homogenates as compared with the radioactivity injected into the mouse brain.

ted, the counts in the total homogenates of the cerebel were taken as 100% and those of the fractions expressed relative to these.

Lysine penetrated the subcellular particles most rapidly and [14 C]norleucine most rapidly. [35 S]taurine also accumulated in the synaptosomes, but remained in the soluble cytoplasm and penetrated the synaptic vesicles and intraterminal mitochondria extremely slowly. Only a very small portion of the [35 S]taurine was attached to the myofibrin and synaptic vesicles. [14 C]glutamate reached all the subcellular fractions, but was present in the mitochondria in relatively low amounts. [14 C]norleucine occurred in all the fractions, but in very minute quantities except in the synaptosomal fractions, where it occurred in amounts similar to taurine. The percentage recovery rates for the amino acids, as calculated from the label in the total homogenates of the cerebel, were about 21% for [35 S]taurine and lysine, but significantly less for [14 C]glutamate and [14 C]norleucine (Table II).

Discussion

Although there are several possible sources of error associated with a study like that reported here, the experiments nevertheless clearly demonstrate penetration of various subcellular fractions by labelled amino acids when injected intracerebrally. The difficulties encountered in the intracerebral injection of amino acids may be caused by the different types of distribution possible in brain matter. One portion will certainly fall into the blood capillaries and be rapidly eliminated through the circulation, another portion may reach the brain ventricles and be diluted by distribution into the brain-cerebrospinal fluid, one portion may perhaps fall into the extracellular space, and another directly into the neurons and glial cells. This may be compensated for, however, by the use of several animals in one experiment.

Relatively clear and interesting differences in distribution could be observed between the four amino acids. The four amino acids used are of different metabolic and neurobiological types. 1) taurine, for which a neurotransmitter or modulator role has been proposed (Kaczmarek and Davison 1972, Oja and Lähdesmäki 1974), but which is metabolically very stable (Lähdesmäki and Korhonen 1978), 2) lysine, which metabolizes slowly and which is known to occur in synaptic vesicles (Lähdesmäki and Winter 1977), 3) glutamic acid, which is apparently a neurotransmitter with effective transport systems into the synaptosomes (Lähdesmäki *et al.* 1975) and synaptic vesicles (Lähdesmäki *et al.* 1977 a)

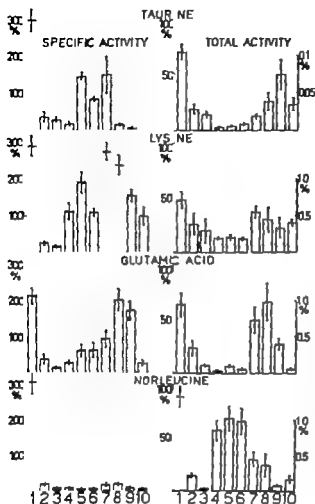


Fig. 2. Distribution of radioactivity by subcellular fractions after intracerebral injection of [3 S]-taurine, [3 S]-lysine, [3 C]-glutamate and [3 C]-norleucine in mouse brain subcellular fractions in terms of specific activity (counts min^{-1} μg protein) and total activity (counts min^{-1} original wet weight). The counts obtained for the total homogenates of the cerebral amino acid were taken as 100 and for the other fractions expressed relative to it: 1=soluble cytoplasm, 2=crude nuclei, 3=crude mitochondrial fraction, 4=myelin, 5=synaptosomes, 6=mitochondria, 7=soluble synaptosomes, 8=synaptic vesicles, 9=synaptic membranes, 10=terminal mitochondria. Results from 3 S.D. indicated by vertical bars) are the experiments with 15-20 animals in each.

ment of radioactivity each fraction was dissolved in 0.1 M NaOH mixed in scintillation liquid of Triton X 100 and 60% toluene containing 6 M 2,5-diphenylterazol and 150 mg/l p-bis(2,5-phenylzoly)benzene and then counted on Wallac liquid scintillation spectrometer (ULTRABETA 12M) described previously (Lähdesmäki *et al.* 1977 b).

In order to eliminate the effect of the label distributed in the subcellular fractions in consequent homogenization, the following control experiment was also performed. The cerebral of untreated mice were removed and the corresponding amount of labelled amino acids injected in the earlier experiments added to the homogenizing medium and homogenization and fractionation performed as above. The label distributed in the subcellular fractions in these latter experiments was then subtracted from the obtained in the earlier experiments to obtain real counts for the radioactivity which had penetrated the cell membranes.

Results

The distribution of the radioactivity of the amino acids among the subcellular fractions indicated in Fig. 1 is depicted in Fig. 2 in the form of specific activity (counts min^{-1} μg protein) and total activity (counts min^{-1} g original wet weight), the counts for the control expts. having first been subtracted from the values obtained. In order to eliminate the effect of any variation between the different amino acids in the amounts of radioactivity

1. Recovery rates of the intracerebrally injected amino acids.

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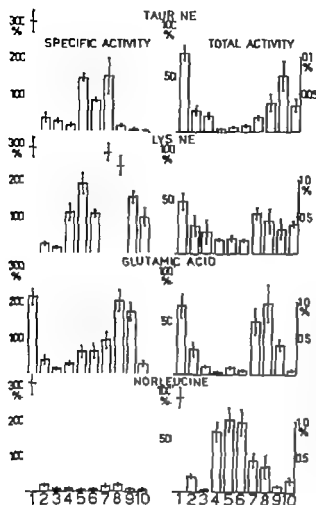


Fig. 2. Distribution of radioactivity in intracerebral injection of ^{14}C -taurine, ^{14}C -lysine, ^{14}C -glutamate and ^{14}C -norleucine in the mouse brain subcellular fractions in terms of specific activity (counts min^{-1} μg protein) and total activity (counts min^{-1} original wet weight). The counts obtained from the total homogenates of the cerebral amino acid were taken as 100 and then the other fractions expressed relative to it. 1 = soluble cytoplasm, 2 = crude nuclear fraction, 3 = crude mitochondrial fraction, 4 = myelin, 5 = synaptosomes, 6 = mitochondria, 7 = soluble synaptoplasm, 8 = synaptosomes, 9 = synaptic membranes, 10 = terminal mitochondria. Results (mean \pm S.D., indicated by vertical bar) are from experiments with 15–20 animals in each.

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) and also specific receptor sites at the synaptic membranes (Michaelis *et al.* 1974, Kinnik *et al.* 1977 b). There is strong evidence that glutamate is an excitatory neurotransmitter in the central nervous system (Curtis and Johnston 1974).

tyrosine

tyrosine is a completely inert amino acid in the central nervous system both metabolically and chemically. It does not occur naturally but is not poisonous, for example, and is used in comparative studies. It penetrates the brain cells and cell particles very slowly compared with other amino acids used, and its low recovery rate is indicative of relatively little excretion during the experiment. The blood-brain barrier apparently allows a foreign substance such as norleucine to penetrate easily in both directions.

It thus appears that intracerebrally injected amino acids, and principally those which naturally occur in brain tissue and subcellular particles and which apparently have a neurological action, may be exchanged between the intracerebral and intracellular amino acid pool. This in turn leads to their accumulation in nerve terminals, mitochondria and synaptic vesicles.

This study is supported by the Research Foundation of the Orion Corporation and the National Research Council of the Natural Sciences, Finland.

References

1. H-TAKI, M. B. ROLLAND and P. GUYARD, Pig brain 4-aminobutyrate 2-ketoglutarate transaminase. Purification, kinetics and physical properties. *Biochimica* 1974 50 823-832.
2. S. R. and A. LARSEN, Amino acid transport. Ed. A. Larssen. In *Handbook of Neurochemistry* Vol. 7. Plenum Press, New York 1972 543-572.
3. W. D. R. and G. A. R. JOHNSON, Amino acid transporters in the mammalian central nervous system. *Neuroscience* 1974 99 97-103.
4. KILLICK, J. S. and H. F. BRADFORD, Amino acids in synaptic vesicles from mammalian cerebral cortex. A comparison. *J. Neurochem.* 1973 21 441-451.
5. JOHNSON, J. T. and R. D. O'BRIEN, Metabolites of β -hydroxybutyrate bound to synaptic vesicles isolated from cerebral cortex. *J. Neurochem.* 1971 18 963-973.
6. J. T. J. and W. O. MCCORMICK, Pharmacological effects produced by intracerebral injection of tyrosine in the conscious mouse. *Br. J. Pharmacol.* 1957 12 12-13.
7. KINNICK, L. K. and A. N. DAVIDSON, Uptake and release of tyrosine from rat brain slices. *J. Neurochem.* 1972 19 2355-2362.
8. KINNICK, E. V. J. JOHNSON and P. LÄMSÄLÄ, Physicochemical characterization of tyrosine binding to synaptic membranes in the chicken brain. *Int. J. Neurosci.* 1978. In press.
9. KINNICK, P. and K. KORMONEN, Competitive binding of tyrosine to the degradation of GABA and tyrosine in the brain. *J. Neurochem.* 1978 30 5-11.
10. KINNICK, P. and R. WYTER, A peptide containing aspartic acid, glutamic acid and serine in calf brain synaptic vesicles. *Acta chem. scand.* 1977 31 802-806.
11. KINNICK, P. and S. S. OJA, The effect of hypothermia and chlorpromazine on GABA and tyrosine in mouse brain. Eds. R. Bickel, G. S. Chhina and R. Singh. In *Selected Topics in Environmental Biology*. Springer Publications, New Delhi. 1976 173-177.
12. KINNICK, P. M. PÄRÄ and S. S. OJA, Effect of electrical stimulation and chlorpromazine on the uptake and release of tyrosine, γ -aminobutyric acid and glutamic acid in mouse brain synaptosomes. *J. Neurochem.* 1975 25 675-680.
13. KINNICK, P. A. KARPINEN, H. SAARE and R. WYTER, Amino acids in the synaptic vesicle fraction from calf brain. Content, uptake and metabolism. *Brain Res.* 1977 a. 138 295-308.

and also having specific membrane receptors at the synaptic membranes (Lähdesmäki 1977 b) but which is metabolically labile, and 4) norleucine, which is a completely compound in the central nervous system.

Taurine

Taurine penetrated the nerve terminals relatively rapidly but remained in the sol synaptoplasm and penetrated the synaptic vesicles and mitochondria and became attached at the synaptic membranes only very slowly. Earlier studies show taurine to be one of the most abundant amino acids in synaptic vesicles (Lähdesmäki and Oja 1976, Lähdesmäki *et al.* 1977 a, Lähdesmäki and Winter 1977) and it also occurs in mitochondria (Lähdesmäki and Korhonen 1978). Its exchange rate between the synaptoplasm and synaptosomal particles seems to be very slow as it is also between the blood and brain (Oja *et al.* 1977). Similarly it has only 1/20 of the binding sites possessed by GABA at the synaptic membrane (Kumpulainen *et al.* 1978). Apparently all of [35 S]taurine injected remained in this form, due to its extremely slow metabolism in the central nervous system (Lähdesmäki and Korhonen 1978).

Lysine

[3 H]lysine penetrated the mitochondria, nerve terminals and synaptic vesicles most rapidly and was attached in the myelin and synaptic membranes in the largest quantities. Its exchange rate between the soluble cytoplasm and subcellular particles may be rapid, but it also has a high propensity to become bound at the membranes. This may be derived from its basic nature. The concentration of lysine in the synaptic vesicles is of the same order of magnitude as that of glycine and alanine, for example (Lähdesmäki and Winter 1977). Lysine can be incorporated into proteins, but otherwise its metabolism in the central nervous system is relatively slow (Strecker 1970). The similar recovery rates of lysine and taurine in the total homogenates are indicative of their slow metabolism.

Glutamic acid

Glutamic acid is the most rapidly metabolized amino acid in the central nervous system, being incorporated into proteins and several other compounds (including small molecules and peptides). It also produces glutamine and, through decarboxylation, GABA, which is further metabolized to succinate and via the tricarboxylic acid cycle to CO_2 , the latter reaction being reflected in the relatively low recovery rate for glutamate in the brain homogenate. Thus in our experiments the label which was initially in the glutamate here might have been partially located in several other compounds (glutamine, GABA, succinate semialdehyde and succinate) by the end of the experiment. GABA transaminase is located in the mitochondria (Waksman *et al.* 1968, Bloch Tardy *et al.* 1974) and interestingly the concentration of [14 C]glutamate or compounds derived from it in the mitochondria was low in the present case, indicating its rapid metabolism here. Both glutamate, glutamine and GABA, as well as some peptides containing glutamate, occur in synaptic vesicles (Lähdesmäki *et al.* 1977 a, Lähdesmäki and Winter 1977), and glutamate has specific transport systems both in synaptosomes (Lähdesmäki *et al.* 1975, 1977 b) and synaptic vesicles (Lähdesmäki *et al.*

a) and also specific receptor sites at the synaptic membranes (Michaelis *et al.* 1974, Jernsiki *et al.* 1977 b). There is strong evidence that glutamate is an excitatory neurotransmitter in the central nervous system (Curtis and Johnston 1974).

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It thus appears that intracerebrally injected amino acids, and principally those which naturally occur in brain tissue and subcellular particles and which apparently have a neurobiological action, may be exchanged between the intracerebral and intracellular amino acid pool. This in turn leads to their accumulation in nerve terminals, mitochondria and synaptic vesicles.

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References

1. W. TAYLOR, M. B. ROLLAND and P. GONNARD, Pig brain 4-aminobutyrate 2-oxoglutarate transaminase: Purification, kinetics and physical properties. *Biochemistry* 1974, 55, 823-832.
2. J. S. R. and A. LARSEN, Amino acid transport. Ed. A. Lajtha. *Handbook of Neurochemistry* Vol. 7. Plenum Press, New York 1972, 543-572.
3. T. D. R. and G. A. R. JORDON, Amino acid transporters in the mammalian central nervous system. *Brain* 1974, 97, 183.
4. BELLINGER, J. S. and H. F. BRADFORD, Amino acids in synaptic vesicles from *Drosophila* cerebral cortex: A reappraisal. *J. Neurochem.* 1973, 27, 441-451.
5. RAY, J. T. and R. E. O'BRIEN, Metabolism of [H]tyrosine bound to synaptic vesicles isolated from cerebral cortex. *J. Neurochem.* 1971, 18, 963-973.
6. R. T. J. and W. G. MCCORMICK, Pharmacological effects produced by intracerebral injection of tyrosine in the conscious mouse. *Br. J. Pharmacol.* 1957, 12, 12-15.
7. JAMES, L. K. and A. N. DAVISON, Uptake and release of tyrosine from rat brain slices. *J. Neurochem.* 1972, 19, 2355-2362.
8. SCHWARTZ, E. V. J. FORMALE and P. LAMBERS, Physicochemical characterization of tyrosine binding to synaptic membranes in the chicken brain. *Br. J. Neurosci.* 1978, 8, in press.
9. SCHWARTZ, P. and E. KOSKOVIC, Comparative studies on the degradation of GABA and tyrosine in the rat brain. *J. Neurochem.* 1978, 30, 5-11.
10. SCHWARTZ, P. and R. WINTER, A peptide containing aspartic acid, glutamic acid and serine in calf brain synaptic vesicles. *Acta chem. scand.* 1977, B37, 802-806.
11. SCHWARTZ, P. and S. S. OJA, The effect of hypothermia and chlorpromazine on GABA and tyrosine in mouse brain. Eds. B. Bhatia, G. S. Chhina and B. Singh. *In Selected Topics in Environmental Biology* Narayan Publications, New Delhi, 1976, 173-177.
12. SCHWARTZ, P., M. PASCIA and S. S. OJA, Effect of electrical stimulation and chlorpromazine on the uptake and release of tyrosine, γ -aminobutyric acid and glutamic acid in mouse brain synaptosomes. *J. Neurochem.* 1975, 25, 675-682.
13. SCHWARTZ, P., A. KARPINEN, H. SAARE and R. WINTER, Amino acids in the synaptic vesicle fraction from calf brain: Contents, uptake and metabolism. *Brain Res.* 1977, 132, 295-308.

- LÄHDESMÄKI, P., E. KUMPULAINEN, O. RAASAKKA and P. KYRKE, Interaction of taurine, GABA and glutamic acid with synaptic membranes. *J. Neurochem.* 1977 **29** 819-826.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with a Folin phenol reagent. *J. Biol. Chem.* 1951 **193** 265-275.
- MANGAN, J. L. and V. P. WHITTAKER, The distribution of free amino acids in subcellular fractions of guinea pig brain. *Biochem. J.* 1966 **98** 128-137.
- MICHELIS, E. K., M. L. MICHAELIS and L. L. BOYARSKY, High-affinity glutamic acid binding to synaptic membranes. *Biochim. biophys. Acta (Amst.)* 1974 **367** 338-348.
- NORRIS, E. P., R. J. WURTMAN and J. AXELROD, A simple and rapid method for injecting H³-norepinephrine into the lateral ventricle of the rat brain. *Lif. Sciences* 1967 **8** 281-291.
- OJA, S. and P. LÄHDESMÄKI, Is taurine an inhibitory neurotransmitter? *Medical Biol.* 1974 **52** 143.
- OJA, S., I. LEHTINEN and P. LÄHDESMÄKI, Taurine transport rates between plasma and tissues in 3- and 7-day-old mice. *Quart. J. exp. Physiol.* 1976 **61** 133-143.
- RASSIN, D. K., Amino acids as putative transmitters. Failure to bind to synaptic vesicles of guinea pig cerebral cortex. *J. Neurochem.* 1972 **19** 139-148.
- RASSIN, D. K., J. A. STURMAN and G. E. GAULL, Taurine in developing rat brain. Subcellular distribution and association with synaptic vesicles of [³⁵S]taurine in maternal, fetal and neonatal rat brain. *J. Neurochem.* 1977 **28** 41-50.
- STRECKER, H. J., Biochemistry of selected amino acids. Ed. A. Lajtha. In *Handbook of Neurochemistry* Vol. 1 Plenum Press, New York 1970, pp. 173-207.
- WAKSMAN, A., M. K. RUMNYATSKY, K. KURIYAMA and E. ROBERTS, Localization of γ -aminobutyric acid transaminase in mouse brain. *J. Neurochem.* 1968 **15** 351-357.
- WHITTAKER, V. P. and L. A. BARKER, The subcellular fractionation of brain tissue. In: *Special techniques in the preparation of synaptosomes and their component organelles*. Ed. R. Fried. In *Methods of Neurochemistry* Dekker, New York 1972 **2** 1-52.

Interrelationships between skeletal muscle adaptations and performance as studied by detraining and retraining

By

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Abstract

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Effects of 15 days of detraining and 15 days of retraining were studied in 6 well-trained runners. Detraining resulted in significant decreases in the mean activities of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) of 24% and 13%, respectively, but no significant increases in these enzyme activities were seen with retraining. Maximal oxygen uptake ($\dot{V}O_{2\max}$) decreased by 4% with detraining ($p < 0.05$), but increased by similar amount with retraining. Performance time in an isotime submaximal run decreased by 1.5% ($p < 0.05$) with detraining but still averaged 9% below the initial level after retraining. Total heart rate and peak heart rate during the performance run were higher after detraining by 4 and 9 beats per min, respectively ($p < 0.05$). With retraining, these heart rate values were decreased by 7 and 9 beats per min ($p < 0.05$). Blood lactate concentrations after the $\dot{V}O_{2\max}$ test and performance run were approximately 30% lower after detraining and retraining ($p < 0.05$). Muscle fibre areas for three subjects did not change in biopsy samples taken after detraining and retraining. These data suggest that even in periods of detraining there are significant changes in indices of physiological capacity and function which may alter their upper limit of adaptation, and that a longer period of retraining is necessary for adaptation to its original trained state.

Key words: Adaptation, detraining, retraining, muscle enzymes, oxygen uptake, performance.

When an individual undergoes a prolonged endurance training stimulus for a number of years, he or she undergoes marked adaptations in the oxygen transport system (Ekblom and Hermansen 1968, Hock 1977) and in the capacity of the trained skeletal muscles for oxidative metabolism (Allen *et al.* 1977). When compared to untrained control subjects, however, elite distance runners have maximal oxygen uptake ($\dot{V}O_{2\max}$) values that are approximately two fold greater (Gollnick *et al.* 1972, Pollock 1977), but oxidative enzyme activities (e.g. SDH) that may be up to 4 times greater (Gollnick *et al.* 1972, Fink *et al.* 1977). There are other characteristics of trained skeletal muscle besides elevated oxidative enzyme activities. The capillary supply is enhanced in endurance trained muscle (Brodal *et al.* 1977, Nygaard and Nielsen 1978), an adaptation that has been shown to be related to training stimulus (Lindman and Henriksson 1977). Further, in two recent studies, the cross sectional area of muscle

fibres from swimmers (Nygaard and Nielsen 1978) and runners (Jansson and Kaijser 1978) have been reported to be smaller than those found in untrained people, although data from several earlier studies do not show this (Edström and Ekblom 1972, Gofnick *et al.* 1972).

Short term interruptions of year-long continuous training are often experienced by a trained athlete, but the circulatory and metabolic consequences of such short periods of inactivity are not known. This subject has been addressed by Booth (1977), at least from a theoretical perspective, and in two recent studies (Örlander *et al.* 1977, Henriksen and Reitman 1977). However these latter two investigations employed subjects, who had relatively brief (7–10 weeks) training, were sedentary and therefore could hardly be considered as having approached their limit of adaptation.

In the present study male runners at the peak of their training were tested before and after a 15 day period with no training stimulus, and then were re-examined after a subsequent 15 days of retraining. Oxygen transport capacities, skeletal muscle characteristics and performance capabilities were tested in order to gain an insight into the plasticity of limiting adaptations to the absence and re-application of a training stimulus.

Material and methods

Subjects. 6 male runners volunteered to participate in this investigation after being fully informed of the nature and risks of the procedures involved. They averaged 32.5 ± 2.8 (\pm S.E.) years of age, 68.1 ± 1.1 in weight and 176 ± 3 cm in height. Four of the subjects were distance runners who had trained year after year for at least 4 years, whereas the remaining two subjects ran shorter distances (400–1 900 m), but for months preceding these experiments trained in a similar manner to the distance runners. Training consisted of long (15–30 km) continuous runs each day at a submaximal intensity (ca 75% or more of $\dot{V}O_{2\max}$). The subjects employed in this study were considered to have approached, if not reached their limit of adaptation and all the subjects had run the marathon (42.2 km) in times ranging from 2:58–3:17 h.

General protocol. The subjects were initially tested at the peak of their training (before detraining). During the next 15 days, no training was permitted, and for the first 7 days of the detraining period a walking plastic cast was placed on the right leg, immobilizing the muscles of the calf. For the remaining 8 days of detraining, the subjects performed their normal daily activities except that no strenuous physical activity was permitted. Following the second testing period (after detraining, A) the subjects resumed training, attempting to increase the duration and intensity of training as rapidly as they felt possible. 15 days after retraining the subjects were re-tested (after retraining, B). Only in the latter half of the detraining period were the subjects able to tolerate their previous training distances.

As the subjects were fearful of the negative effects of detraining on their future performance only a 15 day period was possible. The casting was used to enhance the effects of a withdrawal from the training stimulus. No adverse effects from this procedure were experienced by any of the subjects in subsequent running tests.

Testing procedures. Testing took place on two consecutive days for each of the phases of the study. On the first day the subjects reported to the laboratory and a percutaneous needle biopsy (Lind 1962) was made in the lateral head of the right gastrocnemius muscle. Following the biopsy a maximal oxygen uptake ($\dot{V}O_{2\max}$) test was performed on a motor driven treadmill. The treadmill speed was 16.1 km h⁻¹ and after 4 min at 0%, the grade was increased by 2%. Increments every 2 min (10 min) (ca. 10 min). On the second day the subjects performed a treadmill run to exhaustion at 16.1 km h⁻¹ at a grade calculated to demand 90% of the initial $\dot{V}O_{2\max}$. For subsequent testing the same speed and grade for each subject was employed. The time to exhaustion in this submaximal run was taken to be an estimate of endurance performance capacity and during these runs the subjects were unaware of their actual time. During the two treadmill tests expired air was collected in Douglas bags, and the volume in a Tissot spirometer and analyzed for O_2 and CO_2 fractions using a Servo LB-2, respectively. Heart rate was determined from ECG tracings made at

ing tests. At the end of the treadmill tests blood sample was obtained from superficial arm vein and analysed for lactate content using fluorimetric procedure (Lowry and Passonneau 1972).

Muscle sample analysis. One portion of muscle tissue from each biopsy sample was quickly frozen in liquid nitrogen and stored at -80°C for later enzyme analyses. The remaining muscle sample was mounted in embedding medium, frozen in isopentane cooled in liquid nitrogen, and stored at -80°C until serial cross sections ($10\ \mu\text{m}$) were cut at 20°C in a microtome. The muscle fibres are classified on the basis of the myofibrillar ATPase reaction at pH 9.4 (Padykula and Herman 1955) using the preincubation techniques described by Brooks and Kaiser (1970). On this basis, fibres were identified as types ST and FTs (Saltin *et al.* 1977). For the determination of muscle fibre composition 297 (180–478, mean 332 ± 50) fibres were counted in each biopsy sample.

Capillary analysis. Capillaries were identified using the histochemical enzyme periodic acid Schiff (arylase-PAS) reaction of Anderson (1975). Where a capillary was cut longitudinally it was counted as one at each cell entry. The capillary density (capillaries per mm^2) was obtained by outlining an area of muscle cross section, without artefacts, on a photograph (magnification 1500 \times) of an arylase-PAS stained section. The total area was determined by planimetry and the number of fibres within that area (approximately 100) as the number of capillaries belonging to the area were counted, and the ratio determined. The area of capillaries around fibres was obtained by determining the mean number of capillaries on the entry of at least 25 fibres of a specific type. The fibre type area per capillary was calculated by dividing the area of each fibre type by the mean number of capillaries bordering that fibre type. These procedures for determining muscle capillarisation have been discussed in detail by Anderson and Henriksson (1977). The total area of at least 25 fibres of each type was determined with a planimeter from photographs of sections stained with the arylase-PAS reagent. Using this technique, the variation is absolute for ST and FT fibres within duplicate samples from the same muscle (within laboratory) for 29 subjects aged 15–71 (Halkner, Krukenstein and Lippmann-Hansen, personal communication).

Enzyme analyses. Muscle samples for enzyme analyses were homogenised at 1:100 dilution in an 0.1 M (methacoline) buffer, pH 7.6 and containing 0.05% bovine serum albumin and 5 mM 2-mercaptoethanol. Total phenylalanine and succinate dehydrogenase (SDH) activities were determined fluorimetrically using procedures given by Coriell *et al.* (1976) and Emswiler *et al.* (1975), respectively. Lactate dehydrogenase (LDH) was assayed fluorimetrically as the direction of pyruvate reduction. An aliquot of the homogenate was added to a cuvette containing 1 pH 7.0 containing lipoamide, 20 mM sodium pyruvate, 1 mM bovine serum albumin 0.02%, and reduced nicotinamide adenine dinucleotide (NADH), 0.01 mM. All enzyme analyses for each subject were determined on the same day and enzyme activities were expressed on the basis of wet weight.

Statistics. The significance of differences between conditions was tested using the paired *t*-test. Differences between conditions were considered significant at the 95% confidence level.

Results

2) **Max test.** Individual data and mean values obtained during the VO_2 max test are shown in Fig. 1. Detraining resulted in a decrease ($p < 0.05$) in VO_2 max of $0.171\ \text{min}^{-1}$ ($4.73\ \text{l}$), and retraining produced a significant increase of $0.151\ \text{min}^{-1}$ ($0.5\text{--}6.6\%$). As no notable body weight changes occurred in the group of subjects during the two phases of the experiment, similar changes with detraining and retraining were obtained when O_2 max was expressed on a relative basis ($\text{ml}\ \text{kg}^{-1}\ \text{min}^{-1}$). Maximal expiratory volumes (max) followed the same pattern as VO_2 max during the experiment as is evidenced by the absence of significant differences among the V_E max. VO_2 max ratios for the three test periods (Fig. 1). Mean maximal heart rates (HR_{max}) recorded during the VO_2 max test decreased by 4 beats per min ($4\ \text{b}\ \text{min}^{-1}$) (3% , $p < 0.05$) when the training stimulus was removed, 15 days after commencing retraining, HR_{max} decreased significantly by 7 b min $^{-1}$. After detraining mean lactate concentration in blood sampled immediately after a treadmill maximum oxygen uptake test was 23% lower ($p < 0.05$) compared to the 1st test. No further changes in lactate concentration were noted following retraining.

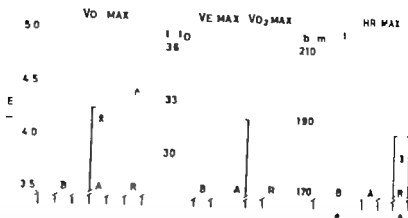


Fig. 1 Means and individual values for maximal oxygen uptake ($\text{VO}_2 \text{ max}$), ventilatory equivalent (VE , $\text{VO}_2 \text{ max}$) and maximum heart rate (HR max), before (B), after 15 days of detraining (A), and after 15 days of retraining (R) for 6 well-trained runners (the symbols \bullet and \square represent subjects who previously trained for 400–1500 m races). — significantly greater than after detraining (A) $p < 0.05$, + significantly less than after detraining (A) $p < 0.05$.

Performance run The work load employed in the treadmill run to exhaustion represents mean energy output that was calculated to be 93.5 (91.9–95.2) % of the initial maximal oxygen uptake. No differences in mean oxygen cost for this run were noted for the three times that the subjects were tested. Performance time (Fig. 2) decreased by 25 (–32 to 50.5) s ($p < 0.05$) with detraining; after retraining it was still 9 (–32 to 30) s below initial time. The peak heart rate recorded during this run increased by 9 b/min ($+10$ to -9) b/min ($p < 0.05$), and in the subsequent test peak heart rate was 9 b/min lower (3–7 b/min, $p < 0.05$). Blood lactate levels obtained after the run were 5.7

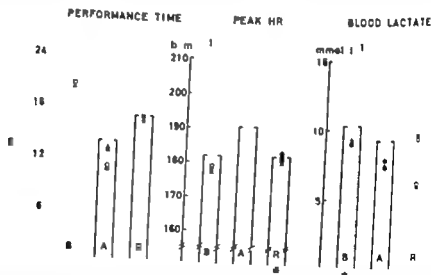
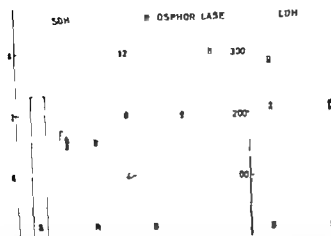


Fig. 2 Means and individual values for performance time in an intense submaximal run, peak heart rate (peak HR) measured during the run and blood lactate concentration measured after the run, before (B), after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners (the symbols \bullet and \square represent subjects who previously trained for 400–1500 m races). — significantly different before detraining (A) $p < 0.05$, + — significantly greater than after detraining (A) and after retraining (R) $p < 0.05$.



Means and individual values for the activities of the enzymes succinate dehydrogenase (SDH), hexokinase and lactate dehydrogenase (LDH) in the lateral part of the right gastrocnemius muscle (B) after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners. Symbols \circ and \square represent subjects who previously trained for 400–1 500 m (max). \circ = significantly lower after detraining (A) and after retraining (R) $p < 0.05$.

SDH activity was higher in the initial test compared to the values obtained after detraining and retraining.

Enzyme activities. Individual values and means for the activities of the enzymes SDH, hexokinase and phosphorylase and LDH are shown in Fig. 3. In the absence of the training stimulus SDH activity declined by 24% ($p < 0.05$), and when training was resumed, SDH activity did not increase significantly. All the subjects but one had notable decreases in SDH activity after detraining. No change in total phosphorylase activity was measured over the phases of the experiment. However mean LDH activity was 13% lower following detraining ($p < 0.05$), and after retraining, LDH activity was 19% lower than the initial value ($p < 0.05$).

muscle fibre composition fibre areas and capillarization. The fibre composition of the 6 subjects at the beginning of the study was $56.1 \pm 6.8\%$ (mean \pm S.E.), $38.2 \pm 3.9\%$ and

Table 1. Fibre composition and fibre areas before (B) and after detraining (A) and following retraining (R) for 3 subjects.

Subject	Fibre composition						Muscle fibre area $\mu m^2 \times 10^3$					
	Type ST			Type FT			Type ST			Type FT		
	B	A	R	B	A	R	B	A	R	B	A	R
1	64.7	61.9	69.1	35.1	36.1	29.5	3.75	4.00	4.84	3.84	4.06	4.56
2	73.6	71.9	72.6	26.0	27.5	27.6	4.06	4.00	5.04	4.85	6.42	7.31
3	52.2	53.2	53.0	47.8	46.2	44.0	4.35	5.56	4.70	3.92	5.18	4.78
4	64.2	61.0	65.1	35.7	36.6	33.7	4.05	4.52	4.69	4.20	5.22	5.56

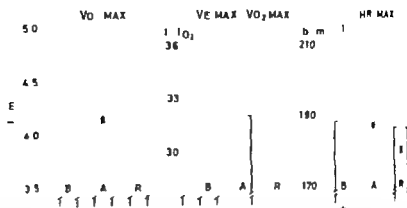


Fig. 1. Means and individual values for maximal oxygen uptake ($\dot{V}O_2$ max), ventilation ($\dot{V}E$ max) and maximum heart rate (HR max), before (B), after 15 days of detraining (A) for 6 well-trained runners (the symbols \bullet and \square represent subjects who were trained for 400-1500 m races). E = significantly greater than after detraining (A) $p < 0.05$; $+$ = significantly greater than after detraining (A) $p < 0.05$; $-$ = significantly less than after detraining (A) $p < 0.05$.

Performance run The work load employed in the treadmill run was a mean energy output that was calculated to be 93.5 (91.9-95.2) kcal/min, which is equivalent to a mean oxygen cost for this run of 3.5 l/min. No differences in mean oxygen cost for this run were found between the times that the subjects were tested. Performance time (Fig. 2) was 50.5 (p < 0.05) with detraining after retraining it was still 50.5 (p < 0.05) initial time. The peak heart rate recorded during this run increased after the test following detraining (p < 0.05), and in the subsequent test it was lower (3-7%, p < 0.05). Blood lactate levels obtained

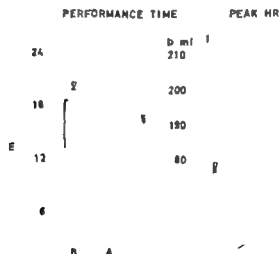
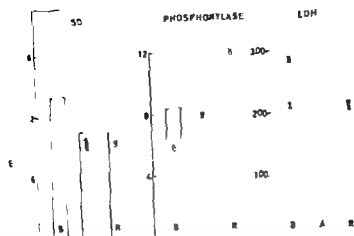


Fig. 2. Means and individual values for performance time and peak heart rate (peak HR) measured during the 1500 m run after 15 days of detraining (A) and before detraining (B). \bullet and \square represent subjects who were trained for 400-1500 m races. E = significantly greater than after detraining (A) $p < 0.05$; $+$ = significantly greater than after detraining (A) $p < 0.05$; $-$ = significantly less than after detraining (A) $p < 0.05$.



enzyme and individual values for the activities of the enzymes succinate dehydrogenase (SDH), hexokinase and lactate dehydrogenase (LDH) in the lateral part of the right gastrocnemius muscle after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners. \circ and \square represent subjects who previously trained for 400–1 500 m (mean). * significantly less after detraining (A) and after retraining (R) $p < 0.05$.

higher in the initial test compared to the values obtained after detraining and retraining.

Enzymes. Individual values and means for the activities of the enzymes SDH, phosphorylase and LDH are shown in Fig. 3. In the absence of the training stimulus SDH activity declined by 24% ($p < 0.05$), and when training was resumed, SDH did not increase significantly. All the subjects but one had notable decreases in SDH activity during detraining. No change in total phosphorylase activity was measured over the course of the experiment. However mean LDH activity was 13% lower following detraining ($p < 0.05$), and after retraining, LDH activity was 19% lower than the initial value ($p < 0.05$).

Fibre composition, fibre areas and capillarization. The fibre composition of the 6 subjects at the beginning of the study was $36.1 \pm 6.8\%$ (mean \pm S.E.), $38.2 \pm 3.9\%$ and

Fibre composition and fibre areas before (B) and after detraining (A) and following retraining (R) for 3 subjects.

Fibre composition %						Muscle fibre area $\mu\text{m}^2 \times 10^3$					
Type ST			Type FT			Type ST			Type FT		
B	A	R	B	A	R	B	A	R	B	A	R
66.7	63.9	69.1	33.1	36.1	29.5	3.75	4.00	4.84	3.84	4.06	4.56
73.6	71.9	72.4	26.4	27.5	27.6	4.06	4.80	5.04	4.85	6.42	7.31
52.2	51.2	53.8	47.8	46.2	44.0	4.35	5.56	4.70	3.92	3.18	4.78
64.2	63.0	65.5	35.7	36.6	33.7	4.05	4.32	4.60	4.20	5.22	5.56

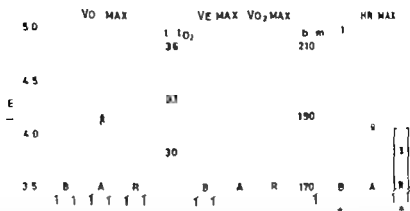


Fig. 1 Means and individual values for maximal oxygen uptake ($\text{VO}_2 \text{ max}$), ventilatory equivalent (VE max), maximal oxygen consumption ($\text{VO}_2 \text{ max}$) and maximum heart rate (HR max), before (B), after 15 days of detraining (A), and after 13 days of retraining (R) for 6 well trained runners (the symbols ● and □ represent subjects who previously trained for 400–1 500 m races). — significantly greater than after detraining (A) $p < 0.05$, + significantly less than after detraining (A) $p < 0.05$.

Performance run The work load employed in the treadmill run to exhaustion represented a mean energy output that was calculated to be 93.5 (91.9–95.2) % of the initial mean oxygen uptake. No differences in mean oxygen cost for this run were noted for the 3 times that the subjects were tested. Performance time (Fig. 2) decreased by 25 (21–50.5) % ($p < 0.05$) with detraining; after retraining it was still 9 (–32 to 30) % below initial time. The peak heart rate recorded during this run increased by 9 b min^{-1} (2–18) the test following detraining ($p < 0.05$) and in the subsequent test peak heart rate 9 b min^{-1} lower (3–7 b min^{-1} , $p < 0.05$). Blood lactate levels obtained after the run were 8

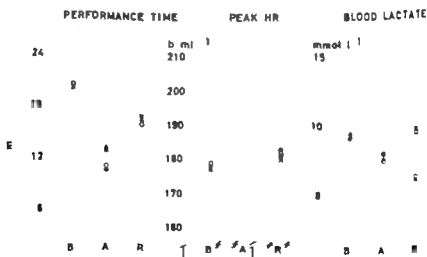
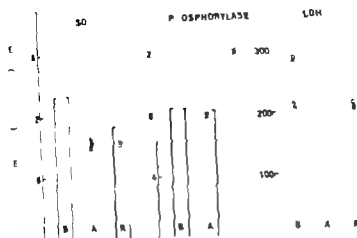


Fig. 2 Means and individual values for performance time in an intense submaximal run, peak heart rate (peak HR) measured during the run and blood lactate concentration measured after the run, before (B), after 15 days of detraining (A) and after 13 days of retraining (R) for 6 well-trained runners (the symbols ● and □ represent subjects who previously trained for 400–1 500 m races). — significantly greater than after detraining (A) $p < 0.05$, + significantly greater than after detraining (A) and $p < 0.05$.



Mean and individual values for the activities of the enzymes succinate dehydrogenase (SDH), isocitrate dehydrogenase (IDH), phosphorylase and lactate dehydrogenase (LDH) in the lateral part of the right gastrocnemius muscle (B) after 15 days of detraining (A) and after 15 days of retraining (R) for 6 well-trained runners. Symbols * and □ represent subjects who previously trained for 400–1 300 m (short). * significantly higher than after detraining (A) and after retraining (R) $p < 0.05$.

higher in the initial test compared to the values obtained after detraining and retraining.

Enzyme activities. Individual values and means for the activities of the enzymes SDH, isocitrate dehydrogenase (IDH), phosphorylase and LDH are shown in Fig. 3. In the absence of the training stimulus SDH activity declined by 24% ($p < 0.05$), and when training was resumed, SDH activity did not increase significantly. All the subjects but one had notable decreases in SDH activity during detraining. No change in total phosphorylase activity was measured over the phases of the experiment. However mean LDH activity was 13% lower following detraining ($p < 0.05$), and after retraining, LDH activity was 19% lower than the initial ($p < 0.05$).

Muscle fibre composition, fibre areas and capillarization. The fibre composition of the 6 subjects at the beginning of the study was $56.1 \pm 6.8\%$ (mean \pm S.E.), $38.2 \pm 3.9\%$ and

1. Fibre composition and fibre areas before (B) and after detraining (A) and following retraining (R) for 6 subjects.

Subject	Fibre composition						Muscle fibre area $\mu\text{m}^2 \times 10^3$					
	Type ST			Type FT			Type ST			Type FT		
	B	A	R	B	A	R	B	A	R	B	A	R
1	66.7	63.9	65.1	33.1	36.1	29.3	3.73	4.00	4.84	3.84	4.06	4.56
2	73.6	71.9	72.4	26.0	27.3	27.6	4.86	4.00	3.04	4.33	6.42	7.31
3	52.2	53.2	53.8	47.8	46.2	44.8	4.35	3.56	4.70	3.92	5.18	4.78
4	64.2	61.0	65.1	35.7	36.6	33.7	4.03	4.32	4.49	4.20	5.22	5.36

TABLE II Muscle capillarization before (B) and after (A) detraining and following retraining (R) in 3 subjects.

Subject	Capillaries per mm ²			Capillaries around fibres						Fibre type area per capillary (mm ²)					
				Type ST			Type FTa			Type ST			Type FTa		
	B	A	R	B	A	R	B	A	R	B	A	R	B	A	R
MH	580	530	570	5.5	5.8	7.0	5.5	5.6	6.8	0.69	0.69	0.62	0.70	0.73	1.0
KR	535	555	481	6.1	6.7	6.7	5.8	8.1	6.9	0.67	0.60	0.75	0.84	0.80	1.0
SK	559	484	500	6.5	6.6	6.5	6.4	6.4	6.5	0.67	0.84	0.72	0.61	0.81	1.0
Mean	558	523	517	6.0	6.4	6.7	5.9	6.7	6.7	0.67	0.71	0.70	0.72	0.78	1.0

$5.5 \pm 3.6\%$ for type ST, FTa and FTb fibres, respectively. Only the two subjects who previously specialized in training for shorter distances had any FTb fibres (13.3 and 19.1) and thus further data for these fibres is omitted. The mean cross sectional area for ST fibres was $4.58 \pm 0.41 \cdot 10^3 \mu\text{m}^2$ and for the FTa fibres, was $5.14 \pm 0.59 \cdot 10^3 \mu\text{m}^2$. There were 525 ± 71 capillaries mm^{-2} and in terms of diffusion, $690 \pm 86 \mu\text{m}^2$ of ST fibre area provided by one capillary; the corresponding value for FTa fibres was $780 \pm 134 \mu\text{m}^2$ capillary.

For the 3 subjects who had been training the longest (approximately 8 years) it was able to obtain complete data on muscle morphology over the course of the experiment. This is illustrated in Table I and II. No significant changes in fibre composition were observed during the two phases of the study although there were some few FTb fibres, representing approximately 0.6% of the total fibre population in the second (A) and third (R) biopsy samples for two of these subjects. The mean areas for ST and FTa fibres in the samples obtained after detraining and retraining tended to be larger compared to the values. However, only for the FTa fibres were the changes in fibre areas greater than expected experimental error. Muscle capillarization (Table II) also appeared to be affected by inactivity and subsequent training. In 2 of the 3 subjects, capillary densities (capillaries per mm^2) were smaller in the second (A) and third (R) biopsy samples.

Discussion

Although the detraining and retraining periods were brief, significant changes occurred in maximal oxygen transport capacity, performance time and muscle enzymes.

Muscle enzymes. Before detraining, the present subjects had muscle SDH activities that were approximately two times higher than is found in untrained males of almost the same age (13.9 vs $7-8 \mu\text{mol g}^{-1} \text{min}^{-1}$) but phosphorylase and LDH activities that were within the range found for the untrained, 8.4 vs $8-12$ and 256 vs $200-300 \mu\text{mol g}^{-1} \text{min}^{-1}$ respectively (unpublished data from this laboratory). The change in the activities of the acid cycle enzyme SDH and the glycolytic enzyme LDH but not the glycogenolytic enzyme phosphorylase would indicate that the activities of the former enzymes in well-trained muscle are dependent in part on the presence of an endurance training stimulus.

Muscle fibre area and capillarization. The tendency to increased fibre areas with detraining

subjects was surprising. The initial fibre areas for these subjects were comparable to values reported by Jansson and Kaijser (1977) for orienteers, and smaller than the mean reported by these authors for the control subjects, that is 4.84 and 5.27 $\times 10^4 \mu\text{m}^2$ for red FTa fibres, respectively. It should be pointed out that although the present data and those reported by Jansson and Kaijser (1977) and Nygaard and Nielsen (1978) reveal that athletes who have undergone prolonged endurance training have small muscle fibres, other studies (Edstrom and Ekblom 1972, Gollnick *et al.* 1972) do not show this. However a physiological consequence of small fibre areas is that diffusion distances for oxygen and other nutrients are reduced. The capillary supply in skeletal muscle has recently been shown to increase in response to exercise training (Andersen and Henriksson 1977) and electrical stimulation (Brown *et al.* 1976). Although only 3 subjects were studied, the present results could suggest that muscle capillarization may be decreased with short detraining. However, further studies are clearly indicated.

Maximal oxygen uptake. The dissociation of changes in whole body oxygen transport capacity from the changes in metabolic oxidative capacity is clearly apparent in this study. The decline in SDH activity with detraining was more than 5 times that for $\text{VO}_{2\text{max}}$. Furthermore, retraining increased maximal oxygen uptake to nearly pre-training values while SDH activity was only marginally increased. These results are in close agreement with those of Henriksson and Reitman (1977) and Ölander *et al.* (1977), and reinforces latter's contention that changes in $\text{VO}_{2\text{max}}$ need not be accompanied by corresponding changes in muscle enzymes.

Performance. The decrease in performance time was of approximately the same relative magnitude as the decrease in SDH activity after detraining. With this in mind, it is tempting to suggest a causal relationship between muscle oxidative capacity as reflected by SDH activity and performance time in the intense submaximal run. However the corresponding values for these two variables after retraining are not related. Rather the decrease in performance time with detraining and the subsequent increase to nearly initial values with retraining are more in accordance with changes in maximal oxygen uptake. At least these results show relative changes that more closely match each other in the direction of change. It may be that the work load selected for the performance measure was too intense to demonstrate a closer relationship with muscle oxidative capacity.

Practical implications. It is not uncommon for man to undergo forced inactivity for brief periods of time as a result of minor injuries. The results of the present study are useful for illustrating the type of changes that could be expected to occur for endurance trained persons. Although the experimental design precludes differentiating between the effects due to the absence of training and those of the casting, some generalizations can be made. First, the inability of the subjects to completely attain initial performance levels following retraining was not very striking. On an average, performance times following retraining were only 9% lower than the initial times and the variation was considerable. The results of this study demonstrate that short lasting detraining (inactivity) leads to moderate reductions in $\text{VO}_{2\text{max}}$ and to relatively greater reductions in submaximal performance times and activities (SDH and LDH). During retraining performance time and in particular $\text{VO}_{2\text{max}}$, are established faster than SDH activity.

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References

- ANDERSEN, P. Capillary density in skeletal muscle of man. *Acta physiol scand.* 1975. 95, 203-205.
- ANDERSEN, P. and J. HENRIKSSON, Capillary supply of the quadriceps femoris muscle of man. Air response to exercise. *J Physiol (Lond)* 1977. 270. 677-690.
- BERGSTRÖM, J. Muscle electrolytes in man. *Scand. J clin Lab Invest.* 1962. Suppl. 68.
- BOOTH, F. Effects of endurance exercise on cytochrome c turnover in skeletal muscle. *Am. N. Y. J. Sci.* 1977. 301. 431-439.
- BRODAL, P., F. INGJER and L. HERMANSEN, Capillary supply of skeletal muscle fibers in trained endurance-trained men. *Amer. J. Physiol.* 1977. 232. H1705-H1712.
- BROOKE, M. H. and K. A. KAISER, Three myosin ATPase systems. The nature of their pH labile sulphhydryl dependence. *J. Histochem. Cytochem.* 1970. 18. 670-672.
- BROWN, M. D., M. A. COTTER, O. HUDLICKÁ and G. VARGOVÁ, The effects of different patterns of activity on capillary density, mechanical properties and structure of slow and fast rabbit muscle. *Arch. gen. Physiol.* 1976. 361. 241-250.
- COSTILL, D. L., J. DANIELS, W. EVANS, W. FINE, G. KRAEMERUTL and B. SALTIN, Skeletal muscle fiber composition in male and female track athletes. *J. appl. Physiol.* 1976. 40. 149-154.
- EDSTRÖM, L. and B. EKBLOM, Differences in sizes of red and white fibres in vastus lateralis of quadriceps femoris of normal individuals and athletes. Relation to physical performance. *Scand. J. Lab. Invest.* 1972. 30. 175-181.
- EKBLOM, B. and L. HERMANSEN, Cardiac output in athletes. *J. appl. Physiol.* 1968. 25. 619-625.
- ESEN, B. E., JANSSON, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN, Metabolic characteristics of types in human skeletal muscle. *Acta physiol scand.* 1975. 95. 153-165.
- FINE, W. J., D. L. COSTILL and M. L. POLLOCK, Submaximal and maximal working capacity of distance runners. Part II. Muscle fiber composition and enzyme activities. *Ann. N. Y. Acad. Sci.* 1971. 301. 323-327.
- GOLLMICK, F. D., R. B. ARMSTRONG, C. W. SAUBERT IV, K. PITHIL and B. SALTIN, Enzyme activity and composition in skeletal muscle of untrained and trained men. *J. appl. Physiol.* 1972. 33. 312-319.
- HENRIKSSON, J. and J. S. RITTMAN, Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity. *Acta physiol. scand.* 1977. 99. 91-99.
- JANSSON, E. and L. KAISER, Muscle adaptation to extreme endurance training in man. *Acta physiol.* 1977. 100. 315-324.
- LOWRY, O. H. and J. V. PASSONNEAU, *A flexible system of enzymatic analysis*. Acad. Press, New York.
- NYGAARD, E. and E. NIELSEN, Skeletal muscle fibre capillarization with extreme endurance training. In *Swimming IV*. Eds. B. Ericsson and B. Froberg. University Park Press, Baltimore. 1971. Press.
- ÖRLANDER, J., K. H. KIERSELOV, J. KARLSSON and B. EKBLOM, Low intensity training, training resumed training in sedentary men. *Acta physiol scand.* 1977. 101. 351-362.
- PADYKULA, H. A. and E. HERMAN, The specificity of the histochemical method of adenosine triphosphatase. *J. Histochem. Cytochem.* 1955. 3. 170-195.
- POLLOCK, M. L., Submaximal and maximal working capacity of elite distance runners. Part I. Respiratory effects. *Ann. N. Y. Acad. Sci.* 1977. 301. 310-322.
- SALTIN, B., J. HENRIKSSON, E. NYGAARD and P. ANDERSEN, Fiber types and metabolic potentials of skeletal muscles in sedentary men and endurance runners. *Ann. N. Y. Acad. Sci.* 1977. 301. 3-29.

Transcapillary passage of albumin, effects of tissue cooling and of increases in filtration and plasma colloid osmotic pressure

By

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Abstract

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Clearance of radiolabelled serum albumin was measured in the perfused, microscopically visualized vascular bed of rat hindquarters during tissue cooling, during increases in filtration and during increases in serum colloid osmotic pressure. Albumin clearance during ordinary serum perfusion at flow rate associated to 0.03 ml/min 100 g, increasing hourly with filtration rate to some 0.07 ml/min 100 g at 0.3 ml/min 100 g of filtration. During cooling from 36°C to 14°C both CFC and lateral clearance of macrovascularly decreased some 40%, in due proportion to the increased viscosity of blood. Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the mean capillary pressure and 'total' albumin clearance during macrovascularly. — It is concluded that during macrovascularly the transmicrovascular albumin passage is to about 70 per cent due to diffusion, and only some 30 per cent of transport at ordinary serum colloid osmotic pressures takes place through, both events presumably via 'large pores'. There was no evidence that transendothelial vesicular transport should be any significant extent contribute to the passage of albumin from vessels to tissues.

The mechanisms behind the transfer of macromolecules across microvascular walls are incompletely understood and a matter of much controversy. While diffusion through small pores is acknowledged as the main principle for transcapillary passage of small solute substances (cf. Landis and Pappenheimer 1963, Crone and Lassen 1970), this is not the case for macromolecules. Grotte (1956) postulated the existence of large capillary 'leaks' through which macromolecules could pass by bulk filtration and/or diffusion. This notion was proposed to be the dominating macromolecular transport mechanism by another group (cf. Shirley *et al.* 1957) and later by Haddy, Scott and Grege (1972, 1976), and, Parving and Rossing (1974) and Ruttili (1977). However, particularly in recent years the interest for endothelial micropinocytosis as a possible major route for transcapillary macromolecular passage has been much increased, especially because of the often suggestive electronmicroscopic illustrations of rapid transport of tracer-labelled macromolecules into the interstitial space and the simultaneous presence of tracer-labelled macromolecules in the interstitial space.

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References

- ANDERSEN, P., Capillary density in skeletal muscle of man. *Acta physiol. scand.* 1975. 95, 203-205.
- ANDERSEN, P. and J. HENRIKSSON, Capillary supply of the quadriceps femoris muscle of man: A response to exercise. *J. Physiol. (Lond.)* 1977. 270, 677-690.
- BERGSTRÖM, J., Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* 1962. Suppl. 68.
- BOOTH, F., Effects of endurance exercise on cytochrome c turnover in skeletal muscle. *Am. J. Physiol.* 1977. 301, 431-439.
- BRODAL, F., F. INGJER and L. HERMANSEN, Capillary supply of skeletal muscle fibers in normal and endurance-trained men. *Amer. J. Physiol.* 1977. 232, H705-H712.
- BROOKE, M. H. and K. K. KAUFER, Three "myosin ATPase" systems. The nature of their pH kinetic and sulfhydryl dependence. *J. Histochem. Cytochem.* 1970. 18, 670-672.
- BROWN, M. B., M. A. COTTER, O. HUDLICKÁ and G. VRAHOVÁ, The effects of different patterns of activity on capillary density, mechanical properties and structure of slow and fast rabbit muscle. *Pflügers Arch. ges. Physiol.* 1976. 361, 241-250.
- COSTILL, D. L., J. DANIELS, W. EVANS, W. FINKE, G. KRAHENBUHL and B. SALTIN, Skeletal muscle fiber type composition in male and female track athletes. *J. appl. Physiol.* 1976. 40, 149-154.
- EDSTRÖM, L. and B. EKBLOM, Differences in sizes of red and white fibers in vastus lateralis of non-athletes and athletes. Relation to physical performance. *Scand. J. Lab. Invest.* 1972. 30, 175-181.
- EKBLOM, B. and L. HERMANSEN, Cardiac output in athletes. *J. appl. Physiol.* 1968. 25, 619-625.
- ESSÉN, B., E. JANSSON, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN, Metabolic characteristics of types in human skeletal muscle. *Acta physiol. scand.* 1975. 95, 133-163.
- FINKE, W. J., D. L. COSTILL and M. L. POLLOCK, Submaximal and maximal working capacity of distance runners. Part II: Muscle fiber composition and enzyme activities. *Ann. N. Y. Acad. Sci.* 1971. 301, 323-327.
- GOLLNICK, P., D. R. B. ARMSTRONG, C. W. SAUBERT IV, K. PIENL and B. SALTIN, Enzyme activities and composition in skeletal muscle of untrained and trained men. *J. appl. Physiol.* 1972. 33, 312-318.
- HENRIKSSON, J. and J. S. REITMAN, Time course of changes in human skeletal muscle succinate dehydrogenase and cytochrome oxidase activities and maximal oxygen uptake with physical activity. *Acta physiol. scand.* 1977. 99, 91-99.
- JANSSON, E. and L. KAUFER, Muscle adaptation to extreme endurance training in man. *Acta physiol. scand.* 1977. 100, 315-324.
- LOWRY, O. H. and J. V. PASSONNEAU, *A flexible system for enzymatic analysis*. Acad. Press, New York.
- NYGAARD, E. and E. NILSEN, Skeletal muscle fibre capillarization with extreme endurance training in man. In *Swimming IV* Eds. B. Ericsson and B. Froberg. University Park Press, Baltimore, MD, 1976.
- ÖRLANDER, J., K. H. KRISTENSEN, J. KARLSSON and B. EKBLOM, Low intensity training, inactive and resumed training in sedentary men. *Acta physiol. scand.* 1977. 101, 351-362.
- PADYKULA, H. A. and E. H. RYAN, The specificity of the histochemical method of adenosine triphosphatase. *J. Histochem. Cytochem.* 1955. 3, 170-195.
- POLLOCK, M. L., Submaximal and maximal working capacity of elite distance runners. Part I: Respiratory effects. *Ann. N. Y. Acad. Sci.* 1977. 301, 310-322.
- SALTIN, B., J. HENRIKSSON, E. NYGAARD and P. ANDERSEN, Fibre types and metabolic potentials of skeletal muscles in sedentary man and endurance runners. *Ann. N. Y. Acad. Sci.* 1977. 301, 3-29.

isogravimetric conditions capillary filtration and absorption are in complete balance, though filtration occurs at the arteriolar end and absorption at the venular end, causing 'pericapillary circulation' (see Pappenheimer 1963).

If the capillary membrane were homogeneous there would be no net solvent drag effects during isogravimetry, net transport being then exclusively diffusive in nature. However according to the two-pore theory (cf Grotte 1954) macromolecules pass almost exclusively through small number of large pores (c. 250 Å), probably located at the venular end of the capillaries (e.g. Wiederhaake 1964). In such pores the reflection coefficient for albumin is likely to be near zero, i.e. there should here be only the 'effective' colloid osmotic pressure gradient to balance off the local hydrostatic pressure gradient. Albumin and other proteins will continuously pass out through the large pores as long as the local static pressure exceeds that in the tissue, and also pass out by diffusion in proportion to the overall difference in protein concentration between perfusate and tissue fluid. At isogravimetry this bulk flow of fluid through the large pores is, however, balanced off by osmotic absorption of solvent through small pores and across endothelial cells, from which macromolecules are excluded (cf Grotte 1954). Such type of isogravimetric volume circulation in the capillary membrane thus includes substantial unidirectional transport of drug transfer of macromolecules from blood to tissue, for which theoretical descriptions have been presented in e.g. Kedem and Katchalsky 1963, Palak and Rapoport 1971, Puri 1973).

In present study the simplicity of these derivations will be tested, i.e. that by Kedem and Katchalsky as modified by Hanes (1974). To simplify the problem it was assumed that the reflection coefficient for proteins were at the large pores, take it as 1 for small pores and endothelial cell membranes. Having expression as then obtained for solvent flux, F_p , of albumin (mg/min 100 g of tissue) and its rate (CL, ml/min 100 g of tissue) (for derivation see appendix I)

$$F = C_p CL \left[\frac{P(C_p - C_t) + \sigma(1 - \sigma)CFC\Delta\pi + (1 - \sigma)CF}{C_p} \right] \quad \text{Eq (2)}$$

C_p the tracer concentration in plasma (mg/ml), C_t the interstitial tracer concentration (mg/ml) and σ the diffusional permeability of albumin through large pores (ml/min 100 g of tissue), σ is the net, weighted osmotic reflection coefficient for serum proteins to the capillary membrane as a whole, assumed to be approximately equal to the weighted solvent drag reflection coefficient (see Appendix I). CFC the capillary filtration coefficient (ml/min mmHg 100 g tissue), $\Delta\pi$ the colloid osmotic pressure difference across the capillary membrane (mmHg), C the mean tracer concentration in the large pores (mg/ml). Finally F represents the net filtration rate (ml/min 100 g tissue).

The first term in the right in Eq (2) represents albumin diffusion through the large pores, the second the osmotic solvent drag transport through the large pores and the last one albumin convective transport coupled to net filtration or absorption. The last-mentioned two terms are derived from the pressure term of Eq 1-9 (see appendix I). The isogravimetric solvent drag term was here derived under the condition of free correction of albumin and most other serum proteins through large pores and complete exclusion from small pores and cell membrane pathways. In reality the isogravimetric drag might be lower because the reflection coefficient for serum proteins in large pores may exceed unity. Further the large pores are probably located at the venular ends of the capillaries where hydrostatic pressure is lower than at the capillary midpoint. The inhomogeneous distribution of large pores is exactly reflected in each represents a weighted mean of all partial reflection coefficients for serum proteins in relation to the different pore structures in the capillary membrane.

As all its limitations, this formula is interesting since it shows how albumin clearance at isogravimetry is partially on the transcapillary colloid osmotic pressure difference ($\Delta\pi$) and also on CFC. Thus, if isogravimetric capillary pressure is increased by raising x_p , the isogravimetric bulk flow of proteins through large pores increases, as does also the osmotic solvent absorption through small pores and endothelial cells. The isogravimetric volume circulation is thus increased and so is the flux of proteins from blood to tissue. As protein clearance by diffusion is independent of concentration, its increase in this case is not coupled to the elevated plasma protein concentration per se but only to the increased isogravimetric volume circulation. Further if CFC is decreased, e.g. by increased fluid viscosity as during isogravimetry, albumin clearance should decrease in proportion to the decrease in CFC and in isogravimetric volume circulation in the capillary membrane.

Even from these considerations it is evident that independent changes of these temperature, of capillary filtration-absorption and of plasma colloid osmotic pressure could be used to discriminate sensitively between diffusive and convective processes involved in albumin transport across endothelial membranes. Vascular transport may here be included among diffusive mechanisms (cf Reekmans 1977), and by means of tissue cooling during isogravimetry or net filtration (cf Rappe, Kaurys and

micropinocytotic vesicles (e.g. Bruns and Palade 1968). Thus, towards the end of such morphologic observations and calculations based on Grotte's experimental data Winne (1965) concluded that macromolecular transfer occurs either by transcapillary vesicular transport or by large pore filtration with only little contribution by other contrast. Renkin and coworkers (cf. Renkin 1964, Garlick and Renkin 1970, Le-Carter and Joyner 1974) considered vesicular transport and large pore diffusion as predominant, but have recently included also large pore filtration, at least during congestion (Renkin *et al.* 1977). Thus, opinions concerning capillary macromolecular transport differ considerably between laboratories, presumably reflecting different methodology and the many technical difficulties involved, as in lymph studies (cf. Le-Carter *et al.* 1977).

Therefore, the aim of the present study was to evaluate to what an extent both convection and vesicular transport contribute to the transvascular albumin passage in the maximally vasodilated isogravimetric rat hindquarter preparation as a model (Folkow *et al.* 1974) since it has proved suitable also for quantitative vascular and capillary flow studies (Rippe and Stage 1978, Rippe, Kamiya and Folkow 1978). After ligation of the lymph drainage the preparation was perfused under well controlled hemodynamic conditions with constant flow with horse serum using radiolabelled albumin as macromolecular tracer. The plasma clearance of tracer albumin could be estimated 1) during tissue cooling which should greatly depress active transport mechanisms 2) during changes in venous pressure and hence in filtration 3) during isogravimetry but at increased plasma colloid osmotic pressures, implying that mean isogravimetric capillary pressure is correspondingly lower. The effect of tissue cooling on the transvascular albumin passage was earlier investigated in paired experiments at equal levels of induced filtration, using a perfusate of lower colloid pressure (Rippe, Kamiya and Folkow 1977). These initial results suggested that capillary micropinocytosis is quantitatively unimportant or even irrelevant as a transport mechanism for large molecules across capillary walls.

Theoretical Considerations

Solvent drag effect on large molecular transport by means of isogravimetric volume circulation in the capillary membrane

The theory of irreversible thermodynamics (Kedem and Katchalsky 1958, 1961, 1963) has recently been applied also for the analysis of transcapillary passage of large molecules (e.g. Perl 1975, Blake and Folkow 1976, Renkin *et al.* 1977). However, even though the data were interpreted in terms of two-pore model, the capillary models, the calculations were generally based on a homogeneous one-pore model, as postulated by Brace, Granger and Taylor (1977). In the present investigation the two-pore capillary model, as postulated by Grotte (1956), was tested applying the simplest derivations concerning irreversible thermodynamics (Kedem and Katchalsky 1963, Hase *et al.* 1974).

According to the Starling hypothesis the capillary fluid equilibrium is a function of the balance between the capillary tissue hydrostatic pressure gradient ($P - P_t$) and the opposing colloid osmotic pressure gradient ($\pi_p - \pi_t$). However, for membranes not perfectly semipermeable this theory should be modified by taking into account the Staverman reflection coefficient (Staverman 1951). According to the theory of irreversible thermodynamics the transcapillary fluid flux is then characterized by:

$$F = CFC[(P - P_t) - \sigma(\pi_p - \pi_t)]$$

where F is the volume flux (ml/min/100 g of tissue), CFC is the hydraulic conductivity (ml/min/100 g) and σ the weighted Staverman osmotic reflection coefficient for the colloid, which is near

and albumin clearance at markedly elevated venous pressures ($P_v > 40$ mmHg)

pairs, perfused at equal temperatures ($36-37^\circ\text{C}$), venous pressure (P_v) in the test preparation was rapidly elevated to 40-70 mmHg while P_v in the control was kept between 3 and 34 mmHg. During the perfusion period the oxygenated heparinized horse serum was diluted 1:60 with Tyrode solution to enhance the filtration. Undiluted horse serum was used during the tracer free perfusion to the preparations isogravimetric under these conditions.

total albumin clearance at different perfusate protein concentrations at normal pressure ($36-37^\circ\text{C}$) and during tissue cooling to $13-15^\circ\text{C}$

Long bovine albumin (Fraction V powder Sigma Chemical Company USA) to normal horse serum, protein concentration was increased from 61 g/l to 111 g/l in two paired experiments and to 86 g/l

in three additional perfusion experiments horse serum, diluted to protein concentration of 36 g/l, was used. In each of these experiments one of the paired preparations was cooled as described above.

Data processing and calculations

Estimated bovine interstitial accumulation of radiolabelled albumin and of tissue fluid was measured at standardized period, usually one hour of tracer perfusion, in order to reach high interstitial tracer concentrations for the measurements, and during ten filtrations. The escape of fluid via lymphatics is neglected (cf. Ruppe and Fölzow 1977). As earlier observed in similar preparations at moderate constant pressure (0.05-0.3 mmHg, 100 g) (e.g. Appelgren, Jacobsson and Kjellner 1966, Friedman 1976), interstitial accumulation of tracer albumin occurs almost linearly during periods up to 1 h. The interstitial protein concentration is determined by the correct balance between "large pore filtration" (in essence restricted passage of plasma) and "small pore filtration" (largely an ultrafiltrate of plasma), which depends on filtration rate and, in turn, on the capillary pressure. Therefore at certain rate of constant filtration in the present study around 0.08 ml/min 100 g during horse serum perfusion these two processes, opposing concentration and the other dilution of interstitial proteins, tend to cancel out so that the albumin accumulation becomes with time perfectly linear function.

On the particular rate of filtration the albumin clearance will after 1 h of tracer perfusion be the same as the tracer perfusion started, and thus reflect the initial albumin clearance. During isogravimetric perfusions, however there will be continuous rise of interstitial colloid concentration with time and the initial albumin clearance value will therefore be underestimated by the one hour net clearance value. The rise of continuous dilution of interstitial colloids, is overestimated after started filtration. This is evident from the theoretical analyses of the transient process of interstitial tracer accumulation, shown in table II. Based on these derivations the 1 h net clearance values were corrected to "initial" clearances as follows: First the 1 h net albumin clearance value CL (ml/min 100 g arterial tissue) is in every experiment calculated from the mean value of the six determinations of specific tracer ^3H in h_1 (CPM/100 g of arterial tissue mean), per unit time, (Δt) and plasma tracer concentration, $P_{3\text{H}}$ ml of plasma):

$$CL = \frac{h_1}{\Delta t P_{3\text{H}}} \quad \text{Eq (3)}$$

By isogravimetry the relation between the initial clearance, CL_0 and CL was calculated from:

$$\frac{CL}{CL_0} = 1 - (\pi P / \Delta \pi) (CL / 2V_0) \quad \text{Eq (4)}$$

where π as earlier defined, $\Delta \pi$ denotes $\Delta \pi$ (see above) at time zero, and where V denotes the initial arterial fluid volume isolated according to Landis and Pappenheimer (1963). π_0 as for undiluted horse serum estimated at 9 mmHg, for 60 diluted horse serum to 10.5 mmHg, for the perfusate containing 86 g protein/l serum and for last containing 111 g protein/l to 60 mmHg. π was according to recent measurements in muscle (Aukland, personal communication) set to 8 mmHg, corresponding to an interstitial protein filtration around 40-50% of that in plasma (cf. Coore, D'Elia and Shaw 1962, Rutli and Arfors 1974, and Aukland 1977). This gives well in line with π_0 estimations in cat muscle (Elamso 1974), if P is around zero. V for albumin distribution is set at 18 ml/100 g muscle as deduced from body measurements of the interstitial fluid space (e.g. Lodgaard-Pedersen and Egeberg 1972).

Folkow 1977) one would be able to discriminate between this active endothelial mechanism and P_A since the latter should be far less temperature-sensitive than microvascular processes (see further). Consequently the present investigation was designed according to these principles, as also described in the Methods.

Methods

General arrangement

Experiments were performed on 22 matched pairs of isolated hindquarters from male Wistar rats weighing between 260 and 350 g. The preparation techniques as well as the general technical arrangement and radioactive assay have earlier been described in detail (Rippe and Folkow 1977). Briefly the experiments were designed as paired, parallel perfusions of one test hindquarter preparation and one control using oxygenated, heparinized horse serum (Normal serum, SBL, Sweden) as perfusate, to which added radiolabelled albumin (^{125}I -albumin Radiocemical Centre, Amersham) as macrocolloid. In the two animals all factors but the test parameters were kept as constant and equal as possible. No vasodilatation was induced and maintained throughout by papaverine infusion, 10–20 $\mu\text{g}/\text{ml}$ perfusate. Continuous recordings were performed concerning mean arterial pressure (P_A in the tail artery), perfusate pressure (P_V in caval side branch), flow rate (8–12 ml/min), total weight and tissue temperature of the preparations, which were arranged for the isogravimetric technique (Pappenheimer and Soto-Rainey 1978).

Subsequent to the period of tracer perfusion, which usually lasted 60 min, the vascular tree was free from tracer during 8 min by shifting to tracer free perfusate. Three pairs of standardized muscle samples (cf. Rippe and Folkow 1977) were then dissected out from each preparation, weighed and analysed with respect to tracer content in a well scintillation counter after which they were dried and assayed. Perfusate content of free ^{125}I was estimated by precipitating the perfusate with 10% TCA and counting supernatant. It averaged 1.6% of total perfusate radioactivity. Correction for free iodine activity, however, not deemed necessary because free iodine is distributed extracellularly and should thus be washed out during the 8 min of final tracer free perfusion.

Tissue fluid accumulation in the musculature was followed by continuous weighing of the net hindquarter on a balance connected to a force transducer (Gram PT 10C) writing on a Gram polygraph. Tissue increase during the perfusion period, as corrected for the skeleton (8% of the hindquarter weight), related well with the calculated weight increase of the muscle samples. The latter values were derived from the relation between wet and dry weights, as compared to corresponding samples from 'non-perfused' controls (Rippe and Folkow 1977).

Determination of perfusate protein concentrations was performed according to the method of Vickers M300 and by paper electrophoresis. The ratio between albumin and globulin concentrations in the perfusate needed for the colloid osmotic pressure estimations, as described under Data processing, was determined by paper electrophoresis.

Experimental procedure

1 Initial albumin clearance during isogravimetry and moderate filtration at normal temperature (36–37°C) and during tissue cooling to 13–15°C

Eight control preparations were perfused at 36°C in parallel with eight test preparations, reduced in temperature to 13–15°C by means of precooling the perfusate to just below 5°C. Two of these preparations were kept isogravimetric throughout, while the others were set to varying rates of filtration. Filtration pressure elevation up to 28 mmHg. In each pair the net filtration rate was kept constant throughout, checked by the weight recordings and slight adjustments. If venous pressure was made whenever necessary. Moreover filtration rates were in most experiments set about equal in both preparations. Tissue temperatures were monitored by thermistors placed in femoral vessels in each hindquarter and corrected by two separate thermoequillibrators (cf. Fig. 1, Rippe and Stage 1978), setting the inflow temperature of the horse serum perfusate to the wished levels. Cooling of the test preparation from 36–37°C to 13–15°C usually required some 15 min, during which period the control preparations were perfused with tracer free perfusate. Before and after the cooling period, CFC (cf. Ellsaesser *et al.* 1974) was measured in four animals. At maximal vasodilatation to check that CFC was reduced roughly in inverse proportion to the temperature dependent viscosity increase, as also observed earlier (cf. Rippe and Gregg 1978). Otherwise CFC measurements were avoided in order not to disturb the capillary fluid equilibrium in preparations kept isogravimetric or at low filtration rates (<0.1 ml/min/100 g).

INITIAL ALBUMIN
CLEARANCE, ml/min 100g

0.30-

60% HORSE SERUM

20-

36°C 36°C
36°C 36°C
36°C 36°C

FILTRATION RATE, ml/min 100g

0 5 10 15 20 25 30 35 40 45 50 55 60 65 70

MEAN VENOUS PRESSURE, mmHg

Initial albumin clearance during perfusion. In horse serum, diluted to 60% with Tyrode, versus mean arterial and venous pressures. At venous pressures below some 40 mmHg there seems to be a linear overlap between albumin clearance at 36°C and filtration rate (-0.97) with approximately the same mass coefficient (β) as for undiluted horse serum at 36°C. Note that the ordinate intercept is only ml/min 100 g for the diluted perfusate, because of the decreased 'isogravimetric' solvent drag rate of albumin at the lower serum colloid osmotic pressure. The regression line above (continuous) calculated from the control values of the six paired experiments, in which the 'test' animals were at the markedly elevated venous pressures, and from the control values of the three paired experiments, in which the 'test' animals were perfused with cooled, diluted horse serum. First two venous pressures above 40 mmHg there is marked increase in albumin clearance, probably reflecting the 'stretched pore' mechanism (cf. Starley *et al.* 1957).

significantly from each other ($p < 0.001$), the ordinate intercepts being 0.0195 ± 0.003 ml/min 100 g and 0.0291 ± 0.0020 ml/min 100 g, respectively. These values represent albumin clearance at isogravimetry in the cooled and control preparations during maximal dilatation. Their difference corresponds closely to the temperature-dependent difference in tissue viscosity which will to the same extent affect isogravimetric large pore filtration. At normal vascular tone the 'effective' exchange area for filtration is 3-4 times lower for diffusion around 4-5 times lower than during maximal vasodilatation (Rippe, 1977 and Foltow 1978). This would imply an albumin clearance value of 0.006-0.007 ml/min 100 g at ordinary resting vascular tone during isogravimetry and normal tissue temperature, in close agreement with *in vivo* values in rabbit skeletal muscle (Bill 1977) and hindpaw (Renkin *et al.* 1977).

Initial perfusate albumin clearance at markedly elevated venous pressure

60% horse serum in Tyrode was used as perfusate the initial albumin clearance extrapolated to isogravimetry during maximal vasodilatation was markedly lower 0.010 ± 0.004

which corresponds well with the albumin space, as deduced from the extravascular mass of it. Interstitial albumin concentration (*cf.* Lassen, Parving and Rosing 1974). This value on V is lower than the one recently obtained by Bell, Watson and Renkin (1978) in skeletal muscle, perhaps reflecting that the muscle interstitial fluid normally forms a variable and easily mobilized fluid depot cardiovascular system (Öberg 1964). Anyway a distribution volume for albumin substantially smaller than 10 ml/100 g of muscle is not compatible with the interstitial pressure-volume data of Ekman *et al.* Using these values for π_p , $\Delta\pi$ and V_p , CL_i^0 can be calculated by Eq (4).

During constant net filtration the initial albumin clearance, CL , can be calculated from the eqn

$$CL = CL_i^0 \left[\frac{\tau_p}{\Delta\pi} \frac{CL}{2F} \frac{\lambda}{1+\lambda} - \left(\frac{\tau_p}{\Delta\pi} - 1 \right) \left(1 - \frac{\ln(1+\lambda)}{\lambda} \right) \right] + CL \quad (5)$$

Here λ equals the total filtered volume ($F \cdot t$), divided by the initial interstitial volume, V ($t = 1$ hr). By inserting the measured values for CL , F and the value for CL_i^0 (see above) the initial albumin clearance during net filtration, CL , can be calculated.

Statistical evaluation was performed according to general parametric routines. Thus, linear regression analysis was carried out as described by Brownlee (1967).

Results

1 Initial serum albumin clearance during isogravimetry and filtration at normal temperature and during tissue cooling to 13–15°C

A significant correlation between initial albumin clearance and filtration rate was also in both the cooled and control preparations, as shown in Fig. 1. The regression coefficients were $(6.33 \pm 1.14) \cdot 10^{-3}$ (\pm S.E.) (dimensionless) and $(7.94 \pm 0.73) \cdot 10^{-3}$ respectively, no significant difference in slope between the two. However, the roughly parallel lines

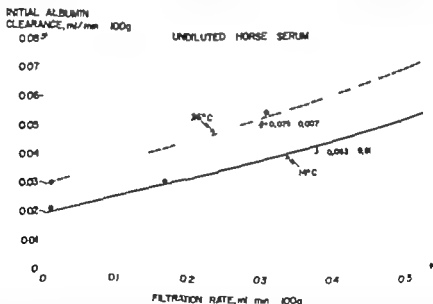
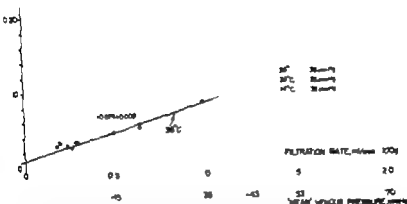


Fig. 1 Initial serum albumin clearance plotted against filtration rate at normal tissue temperature (solid line) and during tissue cooling to 13–15°C (continuous line). Open circles represent clearance values at 16°C and filled circles values at 14°C. Each point represents a mean value of 3 duplicate determinations in every experiment. The roughly parallel lines differ significantly from each other ($p < 0.001$), as isogravimetric albumin clearance was decreased some 35% by cooling. The equations for the lines are $y = 0.0291 + 0.0794x$ ($r = 0.98$) and $y = 0.0195 + 0.0633x$ ($r = 0.91$).

INITIAL ALBUMIN
CLEARANCE, ml/min 100g

60% HORSE SERUM



initial albumin clearance during perfusion with horse serum, diluted to 60% with Tyrode, versus filtration rate and venous pressure. At venous pressures below some 40 mmHg there seems to be a linear overlap between albumin clearance at 36°C and filtration rate ($r=0.97$) with approximately the same mean coefficient (β) as for undiluted horse serum at 36°C. Note that the ordinate intercept is only 0.0195 ml/min 100 g for the diluted perfusate, because of the decreased 'hysteresis' solvent drag seen in albumin at the lower serum colloid oncotic pressure. The regression line shows (conditions calculated from the control value of the unpaired experiments, in which the 'test' animals were subjected to markedly elevated venous pressures, and from the control values of the three paired experiments, in which the test animals were perfused with cooled, diluted horse serum. First at venous pressures above 40 mmHg there is a marked decrease in albumin clearance, probably reflecting the 'stretched pore' mechanism (cf. Starry *et al.* 1957).

significantly from each other ($p < 0.001$), the ordinate intercepts being 0.0195 ± 0.003 ml/min 100 g and 0.0291 ± 0.0020 ml/min 100 g, respectively. These values represent albumin clearance at isogravimetry in the cooled and control preparations during maximal dilatation. Their difference corresponds closely to the temperature-dependent difference in tissue viscosity which will to the same extent affect isogravimetric large pore filtration. At normal vascular tone the effective exchange area for filtration is 3–4 times lower for diffusion around 4–5 times lower than during maximal vasodilatation (Rippe, Wajsbjerg and Følchow 1978). This would imply an albumin clearance value of 0.006–0.007 ml/min 100 g at ordinary resting vascular tone during isogravimetry and normal tissue temperature, in close agreement with the low values in rabbit skeletal muscle (Bill 1977) and hindpaw (Renkin *et al.* 1977).

Initial perfusate albumin clearance at markedly elevated venous pressure

In 60% horse serum in Tyrode was used as perfusate the initial albumin clearance extrapolated to isogravimetry during maximal vasodilatation was markedly lower 0.010 ± 0.004

INITIAL ALBUMIN CLEARANCE
DURING ISOGRAVIMETRY
ml/min 100 g

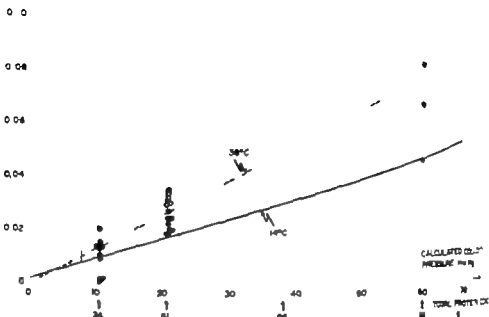


Fig. 3 Initial albumin clearance during isogravimetry plotted against calculated serum colloid pressure (cf Landis and Pappenheimer 1963) at protein concentrations of 111 g/l, 86 g/l and 61 g/l, with data obtained by extrapolation to isogravimetry of clearance above various filtration pressures with 61 g/l and 36 g/l protein concentration. The value on β (see Fig. 1 and 2) despite the extrapolations was 0.072. The Figure shows that the albumin clearance during isogravimetry increases with increasing serum colloid osmotic pressure reflecting an augmented convective transport of it. In connection with increases in isogravimetric capillary pressure. At a serum colloid osmotic pressure of 8 mmHg (vertical dashed line) transcapillary colloid osmotic pressure difference is zero and the isogravimetry is only possible if transcapillary hydrostatic pressure difference were zero. In this situation albumin transport will occur exclusively by dissipative mechanisms. The dissipative component of tracer transport, which at maximal vasodilatation and at the prevailing flow (10 ml/min) should be independent of the level of isogravimetric capillary pressure, was estimated to equal ml/min 100 g.

ml/min 100 g, than when undiluted horse serum was employed (0.029 ± 0.002). The slope coefficient for the relation between albumin clearance and filtration rate was, however, not significantly different from the ones obtained for undiluted serum as long as pressures were kept below 40 mmHg. At venous pressures above some 40 mmHg serum albumin clearance increased markedly as seen from Fig. 2.

3 Initial albumin clearance at different perfusate protein concentrations at normal temperature and during tissue cooling

Using perfusates with different contents of serum protein, the initial albumin clearance values were plotted against perfusate colloid osmotic pressures, calculated according to Landis and Pappenheimer (1963). Fig. 3 presents in such a diagram the albumin clearance values during isogravimetry at protein concentrations of 111 g/l, 86 g/l and 61 g/l, together with clearances for perfusates with 61 g/l and 36 g/l protein concentrations during different rates of filtration, from which the isogravimetric values were extrapolated. It is thus seen that also during isogravimetry albumin clearance increases with increasing perfusate

tic pressure. The clearance values are, however, not only dependent on the transcapillary colloid osmotic difference, because albumin clearance is not zero at plasma colloid pressures identical with those in the interstitial fluid. This is in agreement with eqn 2.

As also seen from Fig. 3 that during tissue temperatures just below 15°C albumin clearance is reduced to about 60 per cent of that during normal tissue temperature of 36–37°C. At the different temperatures the slopes of the linear regression for isogravimetric albumin clearance related to perfusate colloid osmotic pressure are $(1.35 \pm 0.09) \cdot 10^{-4}$ ($r = 0.95$) $(0.80 \pm 0.10) \cdot 10^{-4}$ ($r = 0.90$) respectively.

Discussion

Mechanisms behind the blood to tissue transport of macromolecules has been a matter of controversy over the last few decades, where particularly the possibility that micro-pinocytosis may be involved has aroused much interest. The evidence in support of such an intercellular transport is mainly based on electron microscopic illustrations of tracers readily enter both pinocytotic vesicles and the interstitial fluid. However this fact by no means proves that the tracer is transported to the interstitial space via micro-pinocytosis, it may as well have arrived there by pore-bound filtration and diffusion.

The present investigation is an attempt to estimate in quantitative terms the relative contributions of filtration, diffusion and intercellular transport to the passage of plasma proteins from the vascular bed to the interstitium, using labelled albumin as a tracer in the maximally dilated, perfused rat hindquarter muscle vascular bed. The accumulation of this tracer of fluid in the muscle interstitial fluid space was measured during about one hour of perfusion (cf. Rappé and Folkow 1977) in three different situations, first during tissue cooling, second, at varying filtration rates and, third, at different colloid osmotic pressures of the perfusate. The obtained tracer flux data were mathematically treated to give values for 'actual' albumin clearance, according to derivations presented in appendix II.

As will be further discussed below the results of this study lead no support to the view that microvascular transport should be of any quantitative significance for the transcapillary transport of macromolecules. Rather they strongly suggest that filtration and, to a minor extent, diffusion through large pores is in essence responsible for this macromolecular transfer also during isogravimetry. This conclusion is based on the following findings: 1) During tissue cooling albumin clearance was reduced in close proportion to the temperature-dependent increase of perfusate viscosity and the related decrease in capillary filtration rate, also during isogravimetry. 2) Tracer albumin clearance increased largely in relation to increases in filtration rate. 3) Also during isogravimetry albumin clearance varied in proportion to increases in plasma colloid osmotic pressure.

The data suggesting a filtration rate dependent transport of macromolecules from blood to tissue will first be discussed since similar findings have been earlier presented in numerous (histological) studies (e.g. Szabo, Magyar and Papp 1963, Appelgren, Jacobson and Kjellmer 1964, Haddy, Scott and Greig 1972, Lamen, Parving and Ronborg 1974, Friedman 1976, Folkow et al 1977, Rappé and Folkow 1977). According to the observations by Grotte (1956)

INITIAL ALBUMIN CLEARANCE
DURING ISOGRAVIMETRY
ml/min 100 g

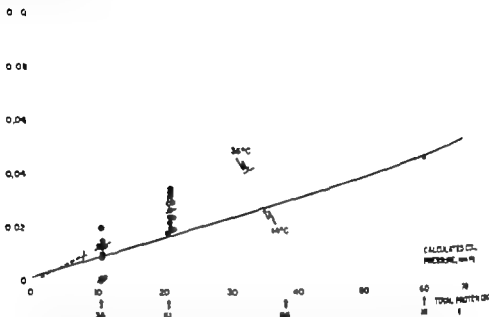


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It is also seen from Fig. 3 that during tissue temperatures just below 15°C albumin clearance is reduced to about 60 per cent of that during normal tissue temperature of 36–37°C. At different temperatures the slopes of the linear regression for biogravimetric albumin clearance as related to perfusate colloid osmotic pressure are $(1.35 \pm 0.09) \cdot 10^{-3}$ ($r = 0.95$) $(0.80 \pm 0.10) \cdot 10^{-3}$ ($r = 0.90$) respectively.

Discussion

Mechanisms behind the blood to tissue transport of macromolecules has been a matter of controversy over the last few decades, where particularly the possibility that microcytosis may be involved has aroused much interest. The evidence in support of such an active cellular transport is mainly based on electron microscopic illustrations of tracers

which enter both pinocytotic vesicles and the interstitial fluid. However, this fact by no means proves that the tracer is transported to the interstitial space via microcytosis, it may as well have arrived there by pore-bound filtration and diffusion.

The present investigation is an attempt to estimate in quantitative terms the relative contributions of filtration, diffusion and vesicular transport to the passage of plasma proteins from the vascular bed into the interstitium, using labelled albumin as a tracer in the maximally perfused, perfused rat hindquarter muscle vascular bed. The accumulation of this tracer in fluid in the muscle interstitial fluid space was measured during about one hour of perfusion (cf. Rippe and Folkow 1977) in three different situations, first during tissue cooling, second, at varying filtration rates and, third, at different colloid osmotic pressures of the perfusate. The obtained tracer flux data were mathematically treated to give values for 'total' albumin clearance, according to derivations presented in appendix II.

As will be further discussed below the results of this study lend no support to the view that microvesicular transport should be of any quantitative significance for the transcapillary passage of macromolecules. Rather they strongly suggest that filtration and, to a minor extent, diffusion through large pores is in essence responsible for this macromolecular transfer also during biogravimetry. This conclusion is based on the following findings: 1) During tissue cooling albumin clearance was reduced in close proportion to the temperature dependent increase of perfusate viscosity and the related decrease in capillary filtration rate, also during biogravimetry. 2) Tracer albumin clearance increased largely in proportion to increases in filtration rate, 3) Also during biogravimetry albumin clearance increased in proportion to increases in plasma colloid osmotic pressure.

The data suggesting a filtration rate dependent transport of macromolecules from blood to tissue will first be discussed since similar findings have been earlier presented in numerous physiological studies (e.g. Szabo, Magyar and Papp 1963, Appellgren, Jacobsson and Kjellmer 1964, Haddy, Scott and Grega 1972, Lassen, Parving and Rossing 1974, Friedman 1976, Folkow *et al.* 1977, Rippe and Folkow 1977). According to the observations by Grotte (1956)

and earlier by White, Field and Drinker (1933), on the other hand, macromolecular transport from blood to lymph seemed to be fairly independent of lymph flow rate. In the present study the initial albumin clearance rose largely in proportion to increases in filtration in all three experimental groups where this relationship was tested, i.e. during tissue perfusate cooling, during perfusion with undiluted horse serum and with diluted horse serum at normal temperature. The coefficient of the linear regression of initial albumin clearance to filtration rate (β) varied between 0.063 and 0.080 (mean 0.072). According to eq 2 (see Theoretical considerations) this would correspond to a mean solvent drag reflection coefficient for albumin (σ_i) of about 0.85–0.86 as deduced from the solvent drag reflection coefficient of the general transport expression of Kedem and Katchalsky (1958). They here assume that the mean tracer concentration in the large pores is about half that in plasma (C_p , $C_p/2$ and $\beta = (1 - \sigma_i)/2$, when the interstitial tracer concentration is near zero.

However the present results indicate that the albumin transport from blood to tissue is largely filtration dependent in which case its concentration in the large pores should be closer to C_p than $C_p/2$ and β closer to $1 - \sigma_i$ than to $(1 - \sigma_i)/2$ (Thomas and Mikulecky, 1969). This implies a solvent drag reflection coefficient for albumin around 0.93 which is in agreement with the osmotic reflection coefficient for plasma proteins obtained by e.g. Paheimer and Soto Rivera (1948) and by Areekul (1969). In the present study it was not possible to determine whether σ_i for albumin is filtration rate dependent, as suggested by Brace, Granger and Taylor (1977) on the basis of theoretical considerations on the two capillary model.

Inserting into Eq 2 the value of 0.93 for σ the mean CFC value for this preparation (0.036 ml/min \cdot 100 g and a transcapillary colloid osmotic pressure difference of 12.5 mmHg (cf. Fadnes and Aukland 1977) gives an initial albumin clearance value of 0.029 ml/min \cdot 100 g, if diffusion transport is set to zero. With a σ value of 0.856 (assuming a mean concentration in the pore of $C_p/2$) approximately the same value on initial albumin clearance (0.028 ml/min \cdot 100 g) is obtained. This value is in good agreement with the experimentally obtained 0.029 ml/min \cdot 100 g. However it should again be pointed out that the theoretically computed value of 0.028–0.029 gives a maximal estimation of the convection contribution to transport, which in reality is likely to be somewhat lower since the term 0.036 ml/min \cdot 100 g must include some contribution by diffusion of albumin, as described by eq 1.

During isogravimetry the passage of tracer albumin from blood to tissue was largely proportional to perfusate colloid osmotic pressure, in good agreement with Eq (2) and supporting the presence of large pore filtration also during isogravimetry (see theoretical considerations). In case the isogravimetric capillary pressure is elevated by increasing perfusate colloid osmotic pressure, this results in an increased unidirectional protein transport by net bulk flow through the large pores. If however large pore filtration were the only mechanism for albumin transfer in the theoretical situation of isogravimetry when perfusate colloid osmotic pressure is lowered enough as to equal that in the interstitial fluid, initial albumin clearance would be zero. This was not the case, as seen from Fig. 3 because the extrapolated initial albumin clearance during isogravimetry at a perfusate colloid osmotic pressure of 8 mmHg was around 0.09–0.10 ml/min \cdot 100 g, or about one third of the clearance at the (for rats) normal perfusate colloid osmotic pressure of around 20 mmHg. This

reflects that there is a non-filtrative component of isogravimetric albumin transport per se with ordinary plasma at maximal vasodilatation, and essentially all of it (theoretical) situation seems to represent diffusion of tracer albumin, as discussed below. Cooling the tissues to 13–15°C by reducing perfusate temperature to below 5°C decreased isogravimetric albumin clearance to about 60% of control, as seen from Fig. 1 and 3. Any permeability characteristics did, however, not change significantly during cooling as would both by CFC measurements, which decreased in due proportion to the temperature-dependent viscosity increase of water, *i.e.* from 0.036 ± 0.001 to 0.024 ± 0.002 ml/min g 100 g, and also by the fact that the solvent drag coefficient (α) for albumin remained about unchanged (Fig. 1). Therefore, the decreases of both CFC and isogravimetric albumin clearances could be satisfactorily ascribed to the predicted increase in viscosity according to Poiseuille's law. Also diffusion decreases in inverse proportion to the viscosity increase and in addition in direct proportion to the decrease in absolute temperature (Stokes-Einstein relation, cf. Landis and Pappenheimer 1963).

The cooling data are incompatible with the concept that vesicular transport should mainly be a major mechanism for macromolecular transport across capillary walls, as strongly suggested by, *e.g.*, Renkin and coworkers (1970, 1974, 1977) and by many morphological experts (*e.g.* Bruns and Palade 1968). Micropinocytosis, a general cell phenomenon occurring also in epithelial cells, fibroblasts, smooth muscle and striated muscle, *etc.* proves to be very sensitive to cooling (Steinman, Silver and Cohn 1974, Allison and Davies 1974).

In fibroblasts the QT_{50} for micropinocytosis is 2.7 (Steinman, Silver and Cohn 1974) implying that micropinocytotic activity at 14°C should be only about 10% of that at 37°C. Isogravimetric albumin clearance during cooling to 14°C was, however, reduced only about 40% of that during normal tissue temperature, which closely fits the temperature-induced reduction of filtration and diffusion capacities.

Therefore, the contribution of vesicular transport to transcapillary passage of albumin, if it in the whole occurs, must be a largely insignificant part of the total dissipative fraction in albumin clearance, which latter during normal plasma perfusion at isogravimetry amounts to about 30% of total clearance. In an earlier study (Rippe and Folkow 1977) cooling hardly at all altered the transcapillary labelled albumin clearance at equal levels of perfusion. The reason is that horse serum diluted to 60% was here used as perfusate and measurements were performed at moderate to high filtration rates, *i.e.* 4–5 ml/min. If the filtration rates and albumin clearance values in that study (around 0.03 ml/min 100 g) are apportioned to isogravimetry using the corresponding value on β of 0.06, an isogravimetric albumin clearance value near to zero is obtained. Thus, the effects of cooling on isogravimetric albumin clearance should, in fact, here be quantitatively almost negligible, because isogravimetric mean capillary pressure is then very low as is also tracer albumin clearance. These circumstances explain the insignificant difference between the theoretically parallel regression lines of albumin clearance *vs.* filtration rate for this diluted perfusate at normal and low perfusions (see above c).

It might perhaps be argued that vesicular transport in capillary endothelium is insensitive to cooling, as suggested on the basis of non-dynamic morphologic data (Jennings and Carey 1967, Basbaum 1973), and that it is also pressure-sensitive (cf. Johansson 1977).

which would seem to invalidate the conclusions made above. However as mentioned in dynamic studies of micropinocytosis show that it is, indeed, highly sensitive to should further be stressed that it is impossible to follow the dynamics of vesicle by purely morphological techniques. For instance, the number of intracellular well be increased during cooling even if micropinocytosis has then completely ceased case exocytosis is slightly more sensitive to cooling than endocytosis. Also the possibility of a pressure dependent micropinocytosis seems highly unlikely as this would imply capillary vesicular transport is in essence unidirectional causing a marked membrane transfer from the luminal to the abluminal side of the endothelial cell, as evident from the following calculation. About 10^4 vesicles of 30 nm radius would be transported from the luminal to the abluminal side per minute to account for the measured isogravimetric albumin clearance of 0.02–0.03 ml/min/100 g. The vesicles needed for such a transport of albumin during one minute represents a capillary membrane area of 1 m²/100 g tissue, which exceeds the total capillary surface area for that amount of muscle tissue (0.7 m²/100 g), according to Leffler and Pappenheimer (1963). This would imply a tremendous turnover of cell membrane which should moreover be largely temperature-insensitive, i.e. independent of cell metabolism to explain the present data. Furthermore, increases in filtration rate to around 1 ml/min/100 g, or a doubling of perfusate colloid osmotic pressure, were found to be the initial albumin clearance. If ascribed to augmented micropinocytosis, the total luminal endothelial membrane would then be transferred to the abluminal side in about 20 s.

Already on the basis of such logistical deductions it is clear that the concept of micropinocytotic transport of macromolecules across the endothelial cells can hardly be sustained, and, further, the present experimental results provide no evidence for this concept. Circulation of bulk flow by means of filtration through large pores, and to a less extent diffusion through these pores, constitute the mechanisms for the normal passage of plasma proteins into the interstitial space. This passage can be greatly accentuated when substances like histamine and bradykinin open up 'venular leaks' a process that is counterbalanced by catecholamines via a β -receptor mediated inhibitory influence (Rippe and Grega 1979).

It remains to be seen which are the true functions of endothelial micropinocytosis, it no doubt reflects some important mechanism. It may, for example, rather represent important microphagocytotic functions, associated with e.g. enzymatic destruction of or removal of blood-borne substances and capillary plugging material, or/and reflect a slow release of blood stream and interstitial fluid of functionally important factors produced by the endothelial cells. It is here interesting that the cerebral capillaries, with very restricted exchange characteristics, show very little of micropinocytotic vesicles. On the other hand, the pulmonary capillaries, mainly designed for rapid transfer of oxygen and carbon dioxide but, like cerebral capillaries, with very limited exchange of water-soluble constituents, show enormous amounts of micropinocytotic vesicles (Waeber 1971). The endothelium of these lung capillaries may well be responsible for the efficient clearance from the blood stream that is observed in the lungs of a number of vasoactive agents like prostaglandins, kinins, and may also accomplish the final elimination of larger particles, which are caught by the 'sieve' function of the pulmonary capillary network. It might be clearing functions like these that are morphologically expressed in terms of the endothelial microvesicles and invagination processes.

ally though further dealt with in a subsequent study the increased albumin permeability occurring first at venous pressures above 40–50 mmHg, also described earlier (e.g. *et al* 1932, Halpern, Musso and Neveu 1955 Renkin *et al* 1977), should be briefly mentioned here. Such high venous pressures are far beyond the physiological bounds for rats and, unlike larger animals are exposed to only small hydrostatic pressure variations upon changes in posture. Nevertheless, there was in the present experiments no evidence of any stretched pore phenomenon even at capillary pressures up towards 50 mmHg, and certainly not at 'physiologic' capillary pressures, though such stretching has been frequently discussed (Weisserman, Loeb and Mayerson (1955) suggested this phenomenon. The exact mechanisms behind the increased microvascular transfer of proteins at markedly elevated venous pressures are not known. Several possibilities must be considered, namely a radius change affecting the small pores or/and a forced opening of preformed large pores, alternative to the more or less irreversible lesional 'rifts' occur throughout the microvascular walls (Rippe, Kamiya and Folkow 1978). These problems will be explored in detail in experiments where also filtration and small molecular diffusion are followed simultaneously. This type of altered microvascular permeability should be clearly distinguished from that seen during capillary exposure to high dextran concentrations (e.g. Kaijser, Lijedahl and Rieger 1969, Areskold 1969, Rippe and Folkow 1977), which at least in part can explain the permeability shifts observed *in vivo* after dextran infusions (e.g. Parving *et al* 1974), earlier attributed to the stretched pore phenomenon.

In summary, the present results, obtained during three principally different test methods, suggest that the main mechanism behind the normal transmicrovascular transfer of albumin is bulk filtration through large pores, even during complete isogravimetry. This occurs in a unidirectional vessel in the transport of plasma proteins by means of an isogravimetric volume circulation in the capillary membrane, which seems to account for some 70% of the albumin transfer and increases in proportion at elevations of isogravimetric capillary pressure. Only around 30% of the albumin transport during isogravimetry at normal plasma colloid osmotic pressure seems to take place by dissipative mechanisms, where diffusion probably dominates and a minor fraction, at most, might be due to a true vesicular transport, for this there is no direct evidence. Further such vesicular transport process should not be mixed up with the possible formation of large pores by means of vesicular confluence (Kronenberg, Semmonescu and Palade 1975), which in that case allows macromolecular transport by filtration and diffusion. Again, there is so far no direct evidence for the formation of such 'transendothelial' large pores to an extent significant for normal capillary transfer processes (Weisig and Williams 1978) though the possibility cannot be excluded. It should anyway in that case not imply any fundamentally different situation from the 'classical' physiological concept of a small pore—large pore population for diffusion-filtration exchange, as outlined in Introduction.

which would seem to invalidate the conclusions made above. However as mentioned in the Introduction, dynamic studies of micropinocytosis show that it is, indeed, highly sensitive to cooling and it should further be stressed that it is impossible to follow the dynamics of micropinocytosis by purely morphological techniques. For instance, the number of intracellular vesicles would be increased during cooling even if micropinocytosis has then completely stopped. In the case of exocytosis is slightly more sensitive to cooling than endocytosis. Also the possibility of a pressure dependent micropinocytosis seems highly unlikely as this would imply that capillary vesicular transport is in essence unidirectional causing a marked membrane transport from the luminal to the abluminal side of the endothelial cell, as evident from the following calculation. About 10 vesicles of 30 nm radius would be transported from the luminal to the abluminal side per minute to account for the measured isogravimetric albumin clearance of 0.02-0.03 ml/min 100 g. The vesicles needed for such a transport of albumin during one minute represents a capillary membrane area of 1 m²/100 g tissue, which exceeds the total capillary surface area for that amount of muscle tissue (0.7 m²/100 g), according to Folkow and Pappenheimer (1963). This would imply a tremendous turnover of cell membrane which should, moreover be largely temperature-insensitive, i.e. independent of cooling, in order to explain the present data. Furthermore, increases in filtration rate to around 0.1 ml/min 100 g, or a doubling of perfusate colloid osmotic pressure, were found to increase the initial albumin clearance. If ascribed to augmented micropinocytosis, the total for the endothelial membrane would then be transferred to the abluminal side in about 26 s.

Already on the basis of such logistical deductions it is clear that the concept of micropinocytotic transport of macromolecules across the endothelial cells can hardly be maintained and further the present experimental results provide no evidence for this concept. Circulation of bulk flow by means of filtration through large pores, and to a less extent diffusion through these pores, constitute the mechanisms for the normal passage of plasma proteins into the interstitial space. This passage can be greatly accentuated when substances like histamine and bradykinin open up venular leaks, a process that is counterbalanced by circulating catecholamines via a β -receptor mediated inhibitory influence (Rippe and Grega 1971).

It remains to be seen which are the true functions of endothelial micropinocytosis, a process which no doubt reflects some important mechanism. It may for example, rather represent important microphagocytotic functions, associated with e.g. enzymatic destruction of toxic blood-borne substances and capillary 'plugging' material or/and reflect a slow release of substances from the blood stream and interstitial fluid of functionally important factors produced by the endothelial cells. It is here interesting that the cerebral capillaries, with very restricted exchange characteristics, show very little of micropinocytotic vesicles. On the other hand, the pulmonary capillaries, mainly designed for rapid transfer of oxygen and carbon dioxide but, like the cerebral capillaries, with very limited exchange of water soluble constituents, show even large amounts of micropinocytotic vesicles (Waeber 1971). The endothelium of these capillaries may well be responsible for the efficient clearance from the blood stream that occurs in the lungs of a number of vasoactive agents like prostaglandins, kinins, and may also be responsible for the final elimination of larger particles, which are caught by the 'sieving' function of the pulmonary capillary network. It might be clearing functions like these that are morphologically expressed in terms of the endothelial microvesicles and invagination

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V the interstitial fluid volume. Provided that PS is relatively small and the two solvent drag effects and the diffusional transport are dominant, the mean concentration of the macromolecular tracer pores, \bar{C} , may be nearly equal to the plasma concentration, C_p (see Discussion).

$$\bar{C} \approx C_p \quad \text{Eq (11-2)}$$

the net filtration rate, F , is unaltered constant, the interstitial fluid volume V is

$$V_t = V + F t \quad \text{Eq (11-3)}$$

water filtration alone dilutes the interstitial fluid but the protein flux simultaneously tends to interstitial colloid osmolarity here their balance determines the final change in π . Since the total amount of protein must be equal to the tracer amount, $(C_t V_t)$, multiplied by the protein/concentration ratio as plasma.

$$\pi_t (V + C_t V_t / C_p) = \pi_p V + \pi_p C_p / C_p \quad \text{Eq (11-4)}$$

π_t π_p at time zero.

derive reasonable correction between the apparent clearance, CL , as measured in the experiments and clearance CL of the tracer the following calculations were performed.

initial clearance CL is theoretically defined as

$$CL = F_p / C_p \text{ at } t=0 \quad (C_p = 0)$$

from Eqs (11-1) and (11-2)

$$CL = PS \cdot \sigma(1 - \sigma) CFC \Delta \pi_0 + (1 - \sigma) F \quad \text{Eq (11-5)}$$

and clearance at no net filtration, CL_1^0 is given by setting $F = 0$ in Eq (11-5).

$$CL_1 = PS \cdot \sigma(1 - \sigma) CFC \Delta \pi_0 \quad \text{Eq (11-6)}$$

the apparent clearance, CL , at time t is given by

$$CL = \frac{1}{t} (V_t C_t - C_p V_0 C_p) = \int_0^t F d(C_p t)$$

following approximate expression for CL for relatively small PS is obtained by integrating Eq (11-1) and,

$$CL = CL_1 + \frac{CL_1^0 \Delta \pi_0}{\Delta \pi_0} \left(\int_0^t C_p dt \right) / (C_p (1 + CL_1^0 (1 - \sigma) \Delta \pi_0 / F) \left(\int_0^t \frac{F t}{F t + V} dt \right) / t \quad (11-7)$$

assuming that

$$\int_0^t C dt = C_p t - CL_1 C_p / F V_0$$

substituting together with

$$\int_0^t \frac{F t}{F t + V} dt = 1 - \frac{\ln(1 + \lambda)}{\lambda} \quad CL = F (1/V_0)$$

Eq (11-7), Eq 5 is obtained. Eq 4 can be obtained from Eq 5 by setting $F = 0$ and writing $CL = CL_1$

References

1. A. C. and P. D. VINT, Mechanisms of endocytosis and exocytosis. *J. sup. Soc. exp. Biol.* 1974, 26, 1-14.
2. L. E. JACOBSON and I. KILLMER, Estimation of the protein concentration of the capillary lumen by an isotopic technique. *Acta physiol. scand.* 1966, 66, 353-363.
3. E. Reflection coefficients of neutral and sulphate-substituted dextran molecules in the isolated hind rabbit ear. *Acta Soc. Med. Scand.* 1969, 74, 129-138.
4. A. C. B. Induced hypertension in peripheral nerve: Electron microscopic and electrophysiological evidence. *J. Neurocytology* 1973, 2, 171-187.
5. D. R. P. D. W. THOM and E. M. REYNOLDS, Exclusion of macromolecules in the interendothelial junctions in dog paw. *Fed. Proc.* 1978, 37, 314.

Appendix I

According to the theory of irreversible thermodynamics, the volume flows through the small and large pores respectively F_{vs} and F_{vl} are given by

$$F_{vs} = CFC_s(\Delta P - \sigma_{cs}\Delta\pi) \quad (1)$$

$$F_{vl} = CFC_l(\Delta P - \sigma_{cl}\Delta\pi) \quad (2)$$

where $CFC_{s,l}$ and $\sigma_{cs,l}$ are the filtration coefficients and the colloid osmotic reflection coefficients small and large pores respectively and, ΔP and $\Delta\pi$ are the hydrostatic and osmotic pressure difference between plasma and interstitial fluid. The total net volume flow F is then

$$F_v = F_{vs} + F_{vl} \quad (3)$$

and consequently the total filtration coefficient CFC and the weighted colloid osmotic reflection coefficient for the whole capillary membrane σ_d are:

$$CFC = CFC_s + CFC_l \quad (4)$$

$$\sigma_d = \sigma_{cs}CFC_s/CFC + \sigma_{cl}CFC_l/CFC \quad (5)$$

Similarly the albumin (tracer) fluxes through the small and large pores F_{as} and F_{al} are

$$F_{as} = PS_s\Delta C + (1 - \sigma_{as})F_{vs}\bar{C}_s \quad (6)$$

$$F_{al} = PS_l\Delta C + (1 - \sigma_{al})F_{vl}\bar{C}_l \quad (7)$$

where $PS_{s,l}$ and $\sigma_{as,l}$ are the permeability coefficient respective the solvent drag reflection coefficient albumin in the small and large pores, ΔC the albumin (tracer) concentration difference between plasma and interstitium and $\bar{C}_{s,l}$ the mean albumin concentration in the small and large pores respectively.

Assuming that the serum proteins freely pass through the large pores but do not pass through the small pores, it follows that,

$$\sigma_{as} \approx \sigma_{al} = 1 \quad \sigma_{cl} \approx \sigma_{pl} = 0 \quad 1 - \sigma_d = \frac{CFC_l}{CFC} \quad P_{as} = 0 \quad F_{as} = 0 \quad \text{and} \quad P_d = P \quad (8)$$

where PS is the weighted albumin (tracer) permeability for the capillary membrane as a whole. Therefore the total solute flux F_s is,

$$F_s = F_{al} = PS_l\Delta C + F_{vl}\bar{C}_l = PS\Delta C + CFC(1 - \sigma_d)\Delta\pi\bar{C}_l \quad (9)$$

Further from Eq (1), i.e.

$$F = CFC(\Delta P - \sigma_d\Delta\pi)$$

we obtain

$$\Delta P = \frac{F + \sigma_d CFC \Delta\pi}{CFC} \quad (10)$$

and substituting Eq (10) into Eq (9),

$$F_s = PS\Delta C + \sigma_d(1 - \sigma_d)CFC\bar{C}_l\Delta\pi + (1 - \sigma_d)\bar{C}_l F \quad (11)$$

Thus, Eq (2) in the text is obtained by rewriting σ_d to σ and \bar{C}_l to \bar{C} . (For more generalized derivation see Houze, 1974.) Notice that in Eq (11), $\Delta\pi$ and σ_d represent the colloid osmotic pressure difference and the weighted osmotic reflection coefficient for the serum proteins while ΔC and \bar{C}_l represent the transcapillary concentration difference respective the mean large pore concentration of albumin (tracer).

Appendix II (A. Kamiya)

From Eq 2 (see theoretical considerations) the transient changes in the interstitial tracer concentration C_i is given by

$$\frac{d(C_i V_i)}{dt} = F - PS(C_p - C_i) + \sigma(1 - \sigma)CFC\Delta\pi\bar{C} + (1 - \sigma)F \bar{C}$$

the interstitial fluid osmotic. Provided that PS is relatively small and the two solvent drag effects as the differential transport are dominant, the mean concentration of the macromolecular tracer in pores, \bar{C}_p , may be nearly equal to the plasma concentration, C_p (see Discussion).

$$\bar{C}_p = C_p \quad \text{Eq (11-2)}$$

At net filtration rate, P_{av} , is maintained constant, the interstitial fluid volume V is

$$V_f = V + F t \quad \text{Eq (11-3)}$$

As water filtration alone will dilute the interstitial fluid but the protein flux simultaneously tends to a interstitial colloid osmolarity where their balance determines the final change in π . Since the local amount of protein must be equal to the tracer amount, $(\bar{C}_p V_f)$, multiplied by the protein/dehydration ratio in plasma,

$$\pi_1 (\pi_p V + C_1 V_p \pi_p / C_p) V = \pi_p V_p V_f + \pi_p C_p V_f \quad \text{Eq (11-4)}$$

π_1 & π_2 at time zero.

Some reasonable correction between the apparent clearance, CL, as measured in the experiments local clearance CL of the tracer the following calculations were performed.

Local clearance CL is theoretically defined as

$$CL = F_p / C_p \quad \text{at } t \rightarrow 0 \quad (C_p = 0)$$

from Eqs (11-1) and (11-2)

$$CL = PS + \alpha(1 - \alpha)CFC\Delta\pi_0 + (1 - \alpha)F \quad \text{Eq (11-5)}$$

local clearance at no net filtration, CL_0 is given by setting $F = 0$ in Eq (11-5).

$$CL_0 = PS + \alpha(1 - \alpha)CFC\Delta\pi_0 \quad \text{Eq (11-6)}$$

The apparent clearance, CL, at time t is given by

$$CL = \frac{1}{t} \int_0^t (C_p - C_1) V_p / (C_p) dt = \int_0^t F dt / (C_p t)$$

Using approximation expression for CL for relatively small PS is obtained by integrating Eq (11-1) as,

$$CL = CL_0 - \frac{CL_0^2 \pi_1 \pi_p \Delta\pi_p}{C_p} \left(\int_0^t C_1 dt \right) / (C_p t) + CL_0^2 (1 - \alpha_p) \Delta\pi_p \left(\int_0^t \frac{F dt}{F t + V} \right) / t \quad (11-7)$$

assuming that

$$\int_0^t C_1 dt = C_p t - CL_0 C_p t V_0$$

substituting together with

$$\int_0^t \frac{F dt}{F t + V} = 1 - \frac{\ln(1 + V)}{V} \quad (V = F t / V_0)$$

Eq (11-7), Eq 5 is obtained. Eq 4 can be obtained from Eq 3 by setting $\pi = 0$ and writing $CL = CL_0$.

References

1. A. C. and P. D. VINE, Mechanism of endocytosis and exocytosis. *Exp. Ser. exp. Biol.* 1974, 20, 1-44.
2. AGAR, L. S. JACOBSON and J. KELLER, Estimation of the protein concentration of the tub by an isotopic technique. *Acta physiol. scand.* 1946, 66, 332-361.
3. ELL, S. Reflection coefficients of neutral and sulphate-substituted dextran molecules in the blood vessel wall. *Acta Soc. Med. Scand.* 1969, 74, 129-132.
4. ALAN, C. B. Isolated hyperthermia in peripheral nerve: Electron microscopic and electrophysiological studies. *J. Neurocytol.* 1973, 2, 171-187.
5. D. R. P. D. W. TROM and E. M. REYNOLDS, Evidence of macromolecules in the interstium of the *in dog per. Fed. Proc.* 1978, 37, 338.

Appendix I

According to the theory of irreversible thermodynamics, the volume flows through the large pores respectively F_{v0} and F_{v1} are given by

$$F_{v0} = CFC_0(\Delta P - \sigma_{00}\Delta\pi) \quad (1)$$

$$F_{v1} = CFC_1(\Delta P - \sigma_{01}\Delta\pi) \quad (2)$$

where $CFC_{0,1}$ and $\sigma_{00,1}$ are the filtration coefficients and the colloid osmotic reflection coefficients small and large pores respectively and, ΔP and $\Delta\pi$ are the hydrostatic and osmotic pressure difference between plasma and interstitial fluid. The total net volume flow F is then

$$F = F_{v0} + F_{v1} \quad (3)$$

and consequently the total filtration coefficient CFC and the weighted colloid osmotic reflection coefficient for the whole capillary membrane σ_0 are:

$$CFC = CFC_0 + CFC_1 \quad (4)$$

$$\sigma_0 = \sigma_{00}CFC_0/CFC + \sigma_{01}CFC_1/CFC \quad (5)$$

Similarly the albumin (tracer) fluxes through the small and large pores F_{a0} and F_{a1} are

$$F_{a0} = PS_0\Delta C + (1 - \sigma_{00})F_{v0}\bar{C}_0 \quad (6)$$

$$F_{a1} = PS_1\Delta C + (1 - \sigma_{01})F_{v1}\bar{C}_1 \quad (7)$$

where $PS_{0,1}$ and $\sigma_{00,1}$ are the permeability coefficient respective the solvent drag reflection coefficient albumin in the small and large pores, ΔC the albumin (tracer) concentration difference between interstitium and $\bar{C}_{0,1}$ the mean albumin concentration in the small and large pores respectively.

Assuming that the serum proteins freely pass through the large pores but do not pass through the small pores, it follows that,

$$\sigma_{00} \approx \sigma_{01} = 1 \quad \sigma_{01} \approx \sigma_{02} = 0 \quad 1 - \sigma_0 = \frac{CFC_1}{CFC} \quad P_{00} = 0 \quad F_{a0} = 0 \quad \text{and} \quad P_{01} = P \quad (8)$$

where PS is the weighted albumin (tracer) permeability for the capillary membrane as a whole \bar{C} the total solute flux F is,

$$F = F_{a1} = PS_1\Delta C + F_{v1}\bar{C}_1 = PS\Delta C + CFC(1 - \sigma_0)\Delta\pi\bar{C}_1$$

Further from Eq (1), i.e.

$$F = CFC(\Delta P - \sigma_0\Delta\pi)$$

we obtain

$$\Delta P = \frac{F + \sigma_0 CFC \Delta\pi}{CFC} \quad (9)$$

and substituting Eq (10) into Eq (1-9),

$$F = PS\Delta C + \sigma_0(1 - \sigma_0)CFC\bar{C}_1\Delta\pi + (1 - \sigma_0)F\bar{C}_1 \quad (10)$$

Thus, Eq (2) in the text is obtained by rewriting σ_0 to σ and \bar{C}_1 to \bar{C} . (For more generalized details see House 1974) Notice that in Eq (11), $\Delta\pi$ and σ_0 represent the colloid osmotic pressure and the weighted osmotic reflection coefficient for all the serum proteins while ΔC and \bar{C}_1 represent transcapillary concentration difference respective the mean large pore concentration of albumin.

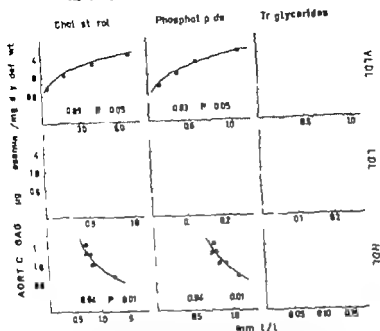
Appendix II (A. Kamiya)

From Eq 2 (see theoretical considerations) the transient changes in the interstitial tracer concentration C_i is given by

$$\frac{d(C_i V_i)}{dt} = F - PS(C_p - C_i) + \sigma(1 - \sigma)CFC\Delta\pi\bar{C} + (1 - \sigma)F\bar{C}$$

2. Effects of cardiovascular reflexes on net capillary fluid transfer. *Acta physiol. scand.* 1964. 62. 229.
3. J. and A. SOTO-RIVERA, Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the bloodvessels of cats and dogs. *Amer. J. Physiol.* 1949. 71-491.
4. H. H. N. ROMING, B. L. NIELSEN and N. A. LAURSEN, Increased transcapillary escape rate of m. IgG and IgM after acute plasma volume expansion in man. *Amer. J. Physiol.* 1974. 227. 243-250.
5. C. S. and S. I. RABUPOORT, Theoretical analysis of net tracer flux due to volume circulation in mesh-like pores of different size. *J. gen. Physiol.* 1971. 57. 113-124.
6. A friction coefficient, series-parallel channel model for transcapillary flux of nonelectrolytes and *Microvasc. Res.* 1971. 8. 189-193.
7. Correction and presentation of fibrosis between plasma and interstitium. *Microvasc. Res.* 1975. 1-94.
8. E. M. Transport of large molecules across capillary walls. *Physiologist* 1964. 7. 13-23.
9. E. M. R. G. CANNON and W. L. JORNER, Mechanism of sustained action of heparin and bradykinin transport of large molecules across capillary walls in the dog paw. *Microvasc. Res.* 1974. 7. 1.
10. E. M. W. L. JORNER, C. H. SLOOF and P. D. W. TROM, Influence of venous pressure on plasma transport in the dog's paw. Constrictive and dissipative mechanisms. *Microvasc. Res.* 1977. 14. 284.
11. B. and B. FOLCOW, Capillary permeability to albumin in normotensive and spontaneously hypertensive rats. *Acta physiol. scand.* 1977. 101. 72-83.
12. B. and G. J. GRANA, Effects of norepinephrine and cooling on histamine induced changes on capillary permeability in the rat hindquarter vascular bed. *Acta physiol. scand.* 1978. 102. 257-262.
13. B. and L. STACE, An 'on-line' computerized method for repeated, rapid determinations of capillary wall capacity. *Acta physiol. scand.* 1978. 102. 108-115.
14. B. A. KAMRY and B. FOLCOW, Is capillary macrophagocytosis of any significance for the transcapillary transfer of plasma proteins? *Acta physiol. scand.* 1977. 100. 253-260.
15. B. A. KAMRY and B. FOLCOW, Simultaneous measurements of capillary diffusion and filtration rates in alterations in the capillary permeability surface area product (PS) and during shifts in micro-absorption. *Acta physiol. scand.* 1978. 102. S2A. S3A.
16. G. Porecapillary mechanisms for macromolecular transport. Symposium "Lymph circulation 300 Years Reflections" Uppsala 1977. *Acta physiol. scand.* T. to be published.
17. G. and K. E. AEPFEL, Protein concentration in interstitial and lymphatic fluids from the subcutaneous tissue. *Acta physiol. scand.* 1977. 99. 1-8.
18. H. H. C. G. WOLFRAM, K. WASSERMAN and H. S. MAYERSON, Capillary permeability to macromolecules: stretched pore phenomenon. *Amer. J. Physiol.* 1957. 190. 189-193.
19. N. M. SANCHEZ and G. E. PALADE, Permeability of mouse capillaries to small molecules. Evidence for the existence of pinocytotic transendothelial channels. *J. cell. Biol.* 1975. 64. 584-607.
20. J. A. J. The theory of osmotic pressure of osmotic pressure. *Rec. trav. chim.* 1931. 70. 344-352.
21. M. R. M. J. M. SILVER and Z. A. COHEN, Phagocytosis in fibroblasts. Quantitative studies in vitro. *Cell Biol.* 1974. 43. 545-549.
22. G. Z. MASTAR and M. PAPE, Correlation between capillary filtration and lymph flow in venous region. *Acta med. Acad. Sci. Hung.* 1963. 19. 185-191.
23. M. S. R. and M. C. MIZULECKY, Transcapillary solute exchange. A comparison of the Kedem-Katchalsky convection-diffusion equations with the rigorous non-linear equations for this special case. *Microvasc. Res.* 1978. 15. 207-230.
24. B. Physiology of the pulmonary circulation. *Angiology* 1971. 8. 266-284.
25. E. M. K. L. LOOS and H. S. MAYERSON, Capillary permeability to macromolecules. *Circulat. Res.* 71. 3. 594-603.
26. J. C. H. E. FIELD and C. K. DUTCHER, On the protein content and normal flow of lymph from the tail of the dog. *Amer. J. Physiol.* 1933. 103. 34-44.
27. J. H. L. C. A., Dynamics of transcapillary fluid exchange. *J. gen. physiol.* 1968. 52. 295-316.
28. D. The Capillary-permeability biochemical Substances. *Pflügers Arch. ges. Physiol.* 1965. 315. 119-124.
29. E. L. and M. C. WILLIAMS, Permeability of mouse capillaries to macromolecules. *J. cell. Biol.* 1974. 41. 379.

- BILL, A., Plasma protein dynamics. Albumin and IgG capillary permeability extra-vascularly and regional blood flow in unanesthetized rabbits. *Acta physiol. scand.* 1977 101 28-42.
- BLAKE, L. H. and N. C. STAUB, Pulmonary vascular transport in sheep, a mathematical model. *K. Res.* 1976. 12. 197-220.
- BRACE, R. A. D. N. GRANGER and A. E. TAYLOR, Analysis of protein flux data. II. Effect of heteroporosity on estimates of reflection coefficients and PS products. *Microvasc. Res.* 1977 14.
- BROWNLEE, K. A. *Statistical Theory and Methodology* Wiley and Sons Inc., New York. 1961.
- BRUNS, R. R. and G. E. PALADE, Studies on blood capillaries. II. Transport of ferritin molecules in wall of muscle capillaries. *J. cell Biol.* 1968. 37 277-299.
- CREER, R. J. L. D'SILVA and O. M. SHAW, Interfibre fluid from guinea pig muscle. *J. Physiol.* 1962 162 44-53.
- CRONE, C. and N. LASSEN, *Capillary Permeability* Alfred Benzon Symposium II. Munksgaard Copenhagen 1970.
- ELIASSEN, E., B. FOLKOW, S. M. HILTON, B. ÖBERG and B. RIPPE, Pressure-volume characteristics of interstitial fluid space in the skeletal muscle of the cat. *Acta physiol. scand.* 1974. 90. 583-593.
- FADNES, H. O. and K. AUKLAND, Protein concentration and colloid osmotic pressure of interstitia collected by the wick technique — Analysis and evaluation of the method. *Microvasc. Res.* 1977 11-25.
- FOLKOW, B., M. HALLBÄCK, Y. LUNDGREN, L. WEISS, J. ALBRECHT and S. JULIUS, Analysis of the reactivity of series-coupled vascular sections in spontaneously hypertensive rats (SHR). *Am. J. Physiol.* 1974. 90. 634-636.
- FRIDMAN, J. J. Transcapillary protein leakage and fluid movement. Effect of venous pressure. *K. Res.* 1976. 13 275-290.
- GARLICK, D. O. and E. M. RENKEN, Transport of large molecules from plasma to interstitial lymph in dogs. *Amer. J. Physiol.* 1970. 219 1595-1605.
- GROTE, G. Passage of dextran molecules across the blood-lymph barrier. *Acta. physiol. scand.* 1956. 211 1-84.
- HADDY, F. J., J. B. SCOTT and G. J. GREGG, Effects of histamine upon lymph protein concentration flow in the dog forelimb. *Amer. J. Physiol.* 1972. 223 1172-1177.
- HADDY, F. J., J. B. SCOTT and G. J. GREGG, Peripheral circulation. Fluid transfer across the micro-membrane. *Int. Review of Physiology Cardiovascular Physiology* 11 1976. 9 63-109. A. C. Goss and A. W. Cowley (eds).
- HALPERN, B. N., E. MUSSO and TH. NEVEU, Action of the histamine releaser polyvinylpyrrolidone on capillary permeability in dogs. *Brit. J. Pharmacol.* 1955 10 213-229.
- HOUSE, C. R., *Water transport in cells and tissues* Edward Arnold (Publ.) Ltd., London. 1974.
- JENNINGS, M. A. and H. W. FLOREY, An investigation of some properties of endothelium of rat permeability. *Proc. roy. Soc. B* 1967 167 39-63.
- JOHANSSON, B. R., *The microvasculature in skeletal muscle. A electromicroscopic study in relation to the endothelial permeability* Thesis. Götting Götting 1977.
- KAUZER, L., S. Ö. LILJEDAHN and Å. REICHER, Blood volume and plasma proteins. IV. Changes in volume and plasma proteins on administration of macrodex — albumin after operations in man. *Acta. physiol. scand.* 1970. 136. 1-7.
- KEDDEM, O. and A. KATCHALSKY, Thermodynamic analysis of the permeability of biological membranes to electrolytes. *Biochim. biophys. Acta* 1958. 27 229-246.
- KEDDEM, O. and A. KATCHALSKY, A physical interpretation of the phenomenological coefficients of binary permeability. *J. gen. Physiol.* 1961 45 143-179.
- KEDDEM, O. and A. KATCHALSKY, Permeability of composite membranes. Parts 1, 2 and 3. *Trans. Faraday Soc.* 1963 59. 1918-1954.
- LADGAARD-PEDERSEN, H. J. and H. C. ENGELL, A comparison of the distribution volumes of ⁵¹Cr-EDTA in man and nephrectomized dogs. *Scand. J. clin. Lab. Invest.* 1972. 30 267-270.
- LANDIS, E. M. and J. R. PAPPENHEIMER, Exchange of substances through the capillary walls. In *Handbook of Physiology* Sec. 2, Circulation. 1963 Vol. II 961-1034 W. F. Hamilton and P. Dow (eds). Physiol. Soc. Washington D.C.
- LANDIS, E. M., L. JOWAT, M. ANGEVINE and W. EBB, The passage of fluid and protein through the capillary wall during venous congestion. *J. clin. Invest.* 1932. 11 717-734.
- LASSEN, N., H. H. PARRYING and K. ROMERO, Filtration as the main mechanism of overall transcapillary protein escape from the plasma. *Microvasc. Res.* 1974 7 1-19.



Relationships between the concentration of aortic glycosaminoglycans (GAGs) and circulating plasma very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) in rats fed the high cholesterol, olive oil diet for 0-3 weeks.

preliminary biochemical study was carried out to detect early effects of a high cholesterol diet on the intima-media of rat aorta by measuring the concentrations of cholesterol, lipoproteins (GAGs) and collagen. The simultaneous changes in plasma lipoprotein during a three weeks induction period of hypercholesterolemia were recorded to find correlations between the concentrations of lipids in plasma lipoprotein fractions and in connective tissue components.

Materials and methods

Animals.—Dewey and Wistar rats, aged 10-14 weeks, were used. Control rats were kept on standard laboratory diet (Hackley Oy Helsinki, Finland, cholesterol concentration 0.4 g/kg, 11% of total fat, 38% carbohydrate, 38% protein). Experimental rats were fed ad libitum on high cholesterol diet (Kero and Pitkanen 1965, cholesterol concentration 15 g/kg, olive oil 200 g/kg, sucrose 520 g/kg, α -cholesterol 5 g/kg, 38% of total calories fat, 45% carbohydrate, 17% protein). This diet does not alter the weight gain of the rats (Pitkanen 1973) i.e. elevates the concentration of plasma cholesterol to 200 mg/dl in 3 weeks with no further increase in 7 weeks, whereas plasma triglyceride concentration is only slightly affected by the diet (Pitkanen 1973, Pitkanen *et al.* 1976).

Experiment A.—Rats (18) were fed standard laboratory diet or the high cholesterol diet for one week or 22 weeks. Four-five rats from both dietary groups were killed at one week or 22 weeks.

Experiment B.—Rats (17) were fed the high cholesterol, olive oil diet for 0-3 weeks. After 8, 1, 3, 7, 12, 14, 21 days on the diet 4-5 rats were killed. 8 rats were fed the standard laboratory diet. 4 of these rats were killed both after 1 and 3 weeks on the standard diet.

Preparation of the aorta.—Rats were killed by open heart puncture under ether anaesthesia after an overnight fast. The descending thoracic aorta (length 25-35 mm) was dissected and stored at -25°C until

Rapid increase of glycosaminoglycans in the aorta of hypercholesterolemic rats, a negative correlation with plasma HDL concentration

By

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Abstract

TAIMI M., T RÖNNEMAA and J VUOKARI *Rapid increase of glycosaminoglycans in the aorta of hypercholesterolemic rats: a negative correlation with plasma HDL concentration*
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Rats were kept either on a standard laboratory diet or a high cholesterol, olive oil diet for periods from 1 day to 22 weeks. The effect of the high cholesterol, olive oil diet on the concentrations of aortic glycosaminoglycans (GAGs) and collagen in aortic intima-media, were studied and the development of hypercholesterolemia was characterized. The concentration of cholesterol in rat aorta was increased after 22 weeks on high cholesterol, olive oil diet while collagen concentration was not affected. On the control diet, the concentration of aortic sulphated GAG was significantly increased already after one week. On the high cholesterol, olive oil diet the diet increased the formation of a cholesterol-rich very low density lipoprotein (VLDL), decreased high density lipoprotein in (HDL)-associated cholesterol and phospholipids, but had virtually no effect on low density lipoprotein in (LDL)-lipids. The concentrations of VLDL-cholesterol and phospholipids showed positive correlations with the concentration of aortic GAGs ($r = 0.85$ and $r = 0.81$ respectively, $P < 0.05$ for both). Stronger (negative) correlations were found between aortic GAG and HDL-cholesterol and -phospholipids ($r = -0.94$ for both, $P < 0.01$) suggesting that HDL may have an effect on the control of arterial sulphated GAG concentration.

Key words: Aorta, cholesterol, collagen, glycosaminoglycans, sulphated hyaluronic acid, hypercholesterolemia, lipoproteins, high density lipoproteins, low density lipoproteins, very low density lipoproteins.

Epidemiological studies have demonstrated that elevated concentrations of plasma low density lipoproteins (LDL) and very low density lipoproteins (VLDL) are associated with a high risk of atherosclerosis and coronary heart disease (Carlson and Eriksson 1975). On the other hand, a strong correlation has been reported between decreased concentrations of high density lipoproteins (HDL) and coronary heart disease (Barter and Nikkila 1953, Miller and Miller 1975, Gordon et al. 1977). It is thus possible that the metabolism of plasma lipoprotein fractions have different influences on arterial connective tissue metabolism, which is known to be affected during the early phase of atherogenesis (Barnes 1960, Ross and Glomset 1973).

11. Concentration of aortic glycosaminoglycans (GAGs, μg hexosamine/mg dry defatted weight) and constituent lipids (cholesterol) in plasma very low density (VLDL), low density (LDL) and high density lipoproteins (HDL) in rats fed the high cholesterol, olive oil diet.

	Days on high cholesterol, olive oil diet						Days back on standard diet	
	0	1	3	7	14	21	7	21
GAGs	0.75 ± 0.10	1.02	1.26	1.24	1.04	1.44	0.96	0.87
cholesterol	0.46 ± 0.07	1.80	1.63	2.76	2.53	6.87	1.10	0.99
phospholipid	0.15 ± 0.02	0.37	0.28	0.61	0.48	1.14	0.33	0.23
triglyceride	0.41 ± 0.12	0.93	0.70	0.92	0.73	0.60	0.61	0.55
cholesterol	0.85 ± 0.13	0.56	0.80	0.70	0.55	0.84	0.53	0.51
phospholipid	0.23 ± 0.04	0.19	0.20	0.22	0.14	0.22	0.19	0.16
triglyceride	0.18 ± 0.04	0.10	0.14	0.23	0.13	0.10	0.14	0.21
cholesterol	1.28 ± 0.17	0.96	0.74	0.83	0.68	0.73	1.71	1.36
phospholipid	1.07 ± 0.05	0.92	0.80	0.74	0.64	0.77	1.19	0.96
triglyceride	0.15 ± 0.02	0.06	0.08	0.15	0.09	0.11	0.16	0.11

values for each day represent the mean of 4-5 rats, analyzed individually at day 0 (\pm S.E.) and in pools at later points.

ted. The experimental diet did not significantly affect the defatted dry weight of the aorta when calculated per internal surface area (Table I). A slight increase in aortic sterol concentration was found at 22 weeks, while the concentration of collagen was changed (Table I). In contrast to the slow increase in aortic cholesterol the concentration of total GAGs was increased already after one week on the high cholesterol, olive oil diet (Table II). This appeared to be due to an increase in sulphated GAGs since the concentration of hyaluronic acid was not significantly affected by the diet.

Experiment B the aortic GAG-concentration increased within a few days on the high cholesterol, olive oil diet (Table II). Plasma VLDL-cholesterol and -phospholipids increased after 3 weeks, while LDL-lipids were not affected by the diet (Table II). Plasma HDL-cholesterol and -phospholipids decreased immediately after the onset of the diet. The aortic GAG-concentration decreased back to the initial level after restoration of standard diet. The changes in VLDL and HDL were also rapidly reversible (Table II). Statistically significant correlations were found during the three weeks experiment between aortic GAG-concentration and plasma VLDL-cholesterol and -phospholipids ($r = 0.89$ and $r = 0.85$ respectively, $P < 0.05$ for both) and HDL-cholesterol and -phospholipids ($r = -0.94$ for both, $P < 0.01$) (Fig. 1). The equations for the curves drawn for VLDL-cholesterol, LDL-phospholipids, HDL-cholesterol and HDL-phospholipids are $y = 0.94 x^{0.88}$, $y = 0.95 x^{0.88}$, $y = 0.85 x^{1.18}$ and $y = 0.85 x^{1.18}$ respectively.

Discussion

The present lipid diet causes a rapid increase in the concentration of GAGs in rat aorta. The early reaction seems to be rather specific to sulphated GAGs because the concentration of hyaluronic acid, total cholesterol and collagen in the aorta were not significantly

TABLE I Effect of the high cholesterol, olive oil diet on the intima-media of rat aorta. The values are the means \pm S.E. of the number of aortas indicated in parentheses. The dry defatted weight of the tissue is expressed as mg/cm² intimal surface area and the other parameters as mg defatted weight

Parameter	1 week		22 weeks	
	Experimental (5)	Control (5)	Experimental (4)	Control (4)
Dry defatted weight	3.98 \pm 0.04	3.82 \pm 0.11	4.33 \pm 0.21	3.91 \pm 0.10
Collagen	243 \pm 8	245 \pm 10	262 \pm 5	261 \pm 10
Cholesterol	4.64 \pm 0.21	5.38 \pm 0.11	6.76 \pm 0.90	5.86 \pm 0.21
Total GAGs (uronic acid)	2.03 \pm 0.10	1.62 \pm 0.16	1.90 \pm 0.29	1.74 \pm 0.10
Total GAGs (bc osamine)	1.69 \pm 0.06	1.49 \pm 0.09	1.72 \pm 0.23	1.70 \pm 0.10
Hyaluronic acid (hexosamine)	0.59 \pm 0.04	0.63 \pm 0.04	0.56 \pm 0.07	0.61 \pm 0.07
Sulphated GAGs (hexosamine)	1.10 \pm 0.06	0.86 \pm 0.06	1.16 \pm 0.17	0.87 \pm 0.17

$P < 0.05$ control aortas as the reference

analyzed. The aorta was opened longitudinally and the intima-media was stripped off under a microscope. The length and area (width of the rectangular piece of intima-media tissue) were estimated with 0.25 mm accuracy for calculation of the intimal surface area.

Determination of plasma and aortic lipids. Lipids were extracted from EDTA-plasma with diethyl ether-methanol (1:1 v/v) (Folch *et al.* 1957), purified (Svennerholm 1968) and aliquots were taken for the determination of cholesterol (Badzioch and Boccon 1966), neutral glycerides (Carlson 1963) and phospholipids (Bartlett 1959; Svanborg and Svennerholm 1961).

The analysis of different plasma lipoprotein fractions was performed according to Vikari *et al.* (1978).

The intima-media samples were rinsed with cold saline, cut into small pieces and extracted with diethyl ether-methanol (2:1 v/v) for 24 h with vigorous shaking and the extraction was repeated. The combined extracts were used for the determination of total cholesterol.

Determination of collagen and glycosaminoglycans. After lipid extraction the aortic intima-media were lyophilized and their dry weight determined. The samples were digested with pepsin (Thomson) and duplicate aliquots from the resulting suspension were hydrolyzed in 6 N HCl at 130°C for 3 h. After evaporation of HCl the concentration of hydroxyproline was determined (Woessner 1961) to calculate approximate collagen content (Pikkarainen 1968).

The rest of the suspension was centrifuged and then GAGs were precipitated from the supernatant with 1% CPC (cetylpyridinium chloride) in 0.02 M NaCl and purified by ethanol precipitation (Thomson). The ethanol precipitate was dissolved in water and used for the determination of hexosamines (by uronic acids (Blumenkrantz and Asboe-Hansen 1973) or fractionated into hyaluronic acid and chondroitin-6-sulphate (C6S) by precipitating sulphated GAGs with 1% CPC in 0.3 M NaCl.

Correlation analysis and statistical calculations. In order to relate the changes found in the concentration of plasma lipoprotein lipids on one hand and in aortic GAGs on the other regression curves of logarithmic, exponential and power functions were calculated with a Texas Instruments calculator. The correlation coefficients were obtained by using power functions (Fig. 1). Student's *t*-test was used to assess the statistical significance.

Results

In Experiment A the plasma lipid values of rats which had been kept for 22 weeks on a high cholesterol, olive oil diet (cholesterol 7.86 \pm 0.9 vs. 1.15 \pm 0.07, $P < 0.001$; triglycerides 1.52 \pm 0.15 vs. 0.71 \pm 0.03, $P < 0.01$ and phospholipids 0.33 \pm 0.07 vs. 0.21 \pm 0.01, $P < 0.01$) were at the same level as they are at 3 and 7 weeks (Pellinen *et al.* 1973). No macroscopic signs of atherosclerosis were observed.

References

- T and H. BOCKM, The determination of free and esterified cholesterol in blood after separation on-layer chromatography *Chin. chem. Acta* 1966, 13 794-797
- J. P. E. M. RIZZ and H. A. EDER, Protein-lipid relationships in human plasma. *Amer J Med* 11 430-493.
- TT III E., Phosphorus assay in column chromatography *J Biol. Chem.* 1959 234 466-468.
- GRANTZ, N and G. AMON-HANSEN, New method for quantitative determination of uronic acids. *J. Biochem* 1973, 34 434-439
- F. Method for the determination of hexosamines in tissues. *J. Biol. Chem.* 1953, 204 553-563.
- CH, L. A. Determination of serum triglycerides. *J. Atheroscler Res.* 1963, 3, 334-336.
- CH, L. A. and M. ELLISON, Quantitative and qualitative serum lipoprotein analysis. Part 2. Studies in survivors of myocardial infarction. *Atherosclerosis* 1975, 21 435-450.
- CH, K. and M. MURRAY, Effect of low density lipoprotein on proteoglycan synthesis by aorta cells from *Experiments (Basil)* 1978, 34 179-181.
- J., M. LEE and G. H. BLAND STAMLEY, A simple method for the purification of total lipids from all tissues. *J. Biol. Chem.* 1957 226 497-509
- L. C. J. R. W. FALLAT, M. SPADAFORA and P. CLARKSON, Longevity syndromes. *Circulation* 1975, 52 11-272.
- J. T. W. P. CASTELL, M. C. HORTLAND, W. B. KAMMEL and T. E. III WICK, High density lipoprotein as protective factor against coronary heart disease. The Framingham study *Amer J Med* 62 707-714
- W. H. Tissue alteration due to experimental arteriosclerosis. In *Connective Tissue and Aging* Vogel, H. G. Excerpta Medica, Amsterdam. 1973, 22 33.
- M. D. R. H. MOORE and H. Z. MOY, The role of smooth muscle cells in the fibrogenesis of atherosclerosis *Amer J Pathol* 1960 37 377-389.
- NOVA, W. Updated concepts on the role of acid mucopolysaccharides and connective tissue proteins accumulation of lipids, lipoproteins, and calcium in the atherosclerotic plaque. *Exp. molec. Path.* 23 106-120
- T and N. KALANT, Aortic glycosaminoglycans in atherosclerosis and alloxan diabetes. *Canad. J. Med* 1962, 44 249-260
- L. P. H. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. *J. Biol. Chem.* 1972, 247 2607-2613.
- J. J. Pathologie der Arterienwand mit besonderer Berücksichtigung der Glykosaminoglykane. VEB J. G. Volk und Geneschilder, Berlin 1973.
- N. M., G. B. ROSE, H. ORIENTE and A. CALL, Possible relationship between aortic acid mucopolysaccharides and atherosclerosis in experimental atherosclerosis. *Nature (Lond.)* 1965, 207 1206.
- CH, G. J. and M. E. MILLER, Plasma-high-density-lipoprotein concentration and development of atherosclerotic disease. *Lancet* 1975, 1 16-19
- J. E., Studies on the lipid-protein relationship in normal and pathologic sera and the effect of serum on serum lipoproteins. *Scand. J. Clin. Lab. Invest* 1963 5 suppl. 2.
- CH, T. T. Lipids of atherosclerotic tissue. Thesis, University of York, 1973.
- CH, T. T. J. VIKARI and L. NORDEN, Effect of hyperlipidaemic diet and clofibrate treatment on atherosclerotic lesions. *Scand. J. Clin. Lab. Invest* 1976 37 117-122.
- BARNEY, J. The molecular structures of vertebrate skin collagens. *Acta physiol. scand.* 1968, Suppl. 23
- J. O. B. Dietary fats and atherosclerosis in rat and rabbit. *Adv. Cardiol.* 1974, 13 169-182.
- CH, R. W. L. M. LUKAR and L. J. LUKAR, Glycosaminoglycans and arterial disease. S. Karger, Basel, 1975.
- R and J. A. CLOONEY, Atherosclerosis and the arterial smooth muscle cell. *Science* 1973, 180 1332-1335
- J. and L. C. FALLON, Effects of dietary lipids on hepatic protein synthesis and lipid metabolism in the rat. *Amer J Physiol* 1965, 208 E82-E86.
- CH, T. T. and P. A. KURUP, Changes in tissue glycosaminoglycans in rats fed hypercholesterolaemic diet. *Atherosclerosis* 1974, 18 65-77
- CH, S. R., P. DOLAN, R. KARMACHARI-MURTHY and G. B. BERKINSON, Isolation of lipoprotein-mucopolysaccharide complexes from fatty streaks of human aortas. *Atherosclerosis* 1972, 16 95-104.

increased (Table I). There were no signs of macroscopic atherosclerosis detectable nor longer feeding periods. This is in agreement with earlier studies (Renaud 1974) as to which olive oil in combination with cholesterol, sucrose and cholic acid is rather atherogenic in the rat. We consider that the early change in GAGs due to the lipid reflects some kind of homeostatic regulation rather than a pathological accumulation. It is supported by the rapid reversibility of the increase in GAGs, when the standard laboratory diet was restored. However accumulation of metachromatic substances in aorta (Tucker 1971) and increased incorporation of ^{35}S into aortic intima-media (Hassett) are typical signs of developing atherosclerosis in the rat. Vijayakumar *et al.* (1975) reported that an atherogenic diet in the rat causes first an increase in aortic sulphated GAGs and later with progressive lipid infiltration a decrease in their concentration; thus seems that a long term feeding of the possibly more atherogenic diets (Ichida & Kalant 1968, Seethanathan and Kurup 1971) decrease the concentration of aortic sulphated GAGs while a shorter feeding period (Vijayakumar *et al.* 1975) and, as in the present study, a less atherogenic diet (Table I) increases aortic sulphated GAGs. Therefore, increased concentration of sulphated GAGs in the arterial wall is not necessarily an essential feature of an already established atherosclerotic lesion but perhaps makes the still normal artery more susceptible to the disease (Mancini *et al.* 1965).

In a recent paper Ehrlich and Murray (1978) found that the synthesis of sulphated proteoglycans by cultured monkey and rabbit aortic smooth muscle cells was stimulated by increasing the concentration of human LDL in the growth medium. On the other hand, preliminary data demonstrate that human sera with high concentrations of HDL inhibit the synthesis of sulphated GAGs by cultured human aortic smooth muscle cells (unpublished). The present study suggests that an increase in plasma VLDL and a decrease in HDL due to the high cholesterol-olive oil diet has similar effects on the concentration of aortic sulphated GAGs *in vivo*. In previous papers (Ichida and Kalant 1968, Seethanathan and Kurup 1971, Vijayakumar *et al.* 1975) the possible correlations between the concentrations of aortic GAGs and the lipids in various lipoprotein fractions have not been determined. However in an *in vivo* experiment it is difficult to state definitely whether the changes in concentrations of various lipoprotein fractions, and which of them, cause the increase in arterial GAGs. In any case, our results suggest that among the various lipoprotein fractions the concentration of HDL is the best predictor for the early increase in the aortic GAGs.

There are many possibilities how arterial GAGs may be involved in the initiation of atherosclerosis (Kunz 1975, Robinson *et al.* 1975). The ability of certain sulphated GAGs to bind and precipitate LDL and VLDL may lead to the accumulation of cholesterol in the arterial wall with subsequent atherosclerosis (Iversius 1972, Srinivasan *et al.* 1972, Holtz 1976). A high concentration of HDL might inhibit the harmful precipitation by decreasing the amount of sulphated GAGs. This could explain the protective effect of high HDL concentration against atherosclerosis (Glueck *et al.* 1975, Gordon *et al.* 1977).

References

- and H. BOGZON, The determination of free and esterified cholesterol in blood after separation with chromatography. *Chim. clin. Acta* 1966. 13, 794-797.
- F. E. M. ROSE and H. A. EDER, Protein-lipid relationships in human plasma. *Amer J Med.* 1960. 40-492.
- G. R., Phosphorus assay in column chromatography. *J Biol. Chem.* 1959. 234. 466-468.
- ARTZ, N. and G. AMON-HANSEN, New method for quantitative determination of uronic acids. *Biochem.* 1973. 34. 484-489.
- F. Method for the determination of hexosamines in tissue. *J Biol. Chem.* 1953. 204. 553-563.
- L. A. Determination of serum triglycerides. *J Atheroscler Res.* 1963. 3. 334-336.
- L. A. and M. ERICSON, Quantitative and qualitative serum lipoprotein analysis. Part. 2. Studies survivors of myocardial infarction. *Atherosclerosis* 1975. 21. 435-450.
- E. and M. MURRAY, Effect of low density lipoprotein on proteoglycan synthesis by aorta cells in *Experimenta (Basel)* 1978. 34. 179-181.
- M. LEE and G. H. SLOAN STAMLEY, A simple method for the purification of total lipids from tissues. *J Biol. Chem.* 1957. 226. 497-509.
- C. J. R. W. FALLAT, M. SPADAFORA and P. GASTNER, Longevity syndromes. *Cholesterol* 1975. 11-272.
- T. W. F. CASTELL, M. C. HORTLAND, W. B. KAMMEL and T. R. D. WINTER, High density lipoprotein protective factor against coronary heart disease. The Framingham study. *Amer J Med.* 1972. 707-714.
- W. H. Tissue alterations due to experimental arteriosclerosis. I. *Connective Tissue and Aging* ed. H. G. Excerpta Medica, Amsterdam 1973. 22. 31.
- M. H. R. H. MOSE and H. Z. MOSE, The role of smooth muscle cells in the fibrogenesis of arteries. *Amer J Pathol.* 1960. 37. 377-389.
- W. Unified concept on the role of acid mucopolysaccharides and connective tissue protein accumulation of lipids, lipoproteins, and calcium in the atherosclerotic plaque. *Exp. molec. Path.* 21. 106-120.
- T. and N. KALANT, Aortic glycosaminoglycans in atherosclerosis and alloxan diabetes. *Canad. J. Med.* 1968. 46. 249-260.
- P. H. The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. *J Biol Chem.* 1972. 247. 2607-2613.
- J. Pathologie der Arterienwand mit besonderer Berücksichtigung der Glykosaminoglykane. VEB J. Volk und Gesundheit, Berlin 1975.
- M. M. G. B. ROSE, P. OIKARINEN and A. CALL, Possible relationship between aortic acid mucopolysaccharides and species-susceptibility in experimental atherosclerosis. *Nature (Lond.)* 1963. 207. 1206.
- L. G. J. and N. E. MILLER, Plasma-high-density-lipoprotein concentration and development of atherosclerotic heart disease. *Lancet* 1975. i. 16-19.
- A. E., Studies on the lipid-protein relationship in normal and pathologic sera and the effect of pH on serum lipoproteins. *Scand. J. Clin. Lab. Invest.* 1953. 5. suppl. 8.
- ROSE, T. T. Lipids of connective tissue. Thesis, University of Turku, 1971.
- ROSE, T. T. J. VIKARI and L. NORDEN, Effect of hyperlipidemic diet and clofibrate treatment on tyrosine lipids. *Scand J Haematol* 1976. 37. 117-122.
- ROSE, J. The molecular structure of vertebrate skin collagen. *Acta physiol. scand.* 1968. Suppl. 111.
- JO, S. Dietary fats and atherosclerosis in rat and rabbit. *Adv. Cardiol.* 1974. 13. 109-112.
- ROSE, R. W. I. N. LILJA and L. J. LILJA, Glycosaminoglycans and arterial disease. S. Karger, Basel, 1971.
- R. and J. A. GLOMSET, Atherosclerosis and the arterial smooth muscle cell. *Science* 1973. 180. 1332.
- I. and L. C. FALICK, Effects of dietary lipids on hepatic protein synthesis and lipid metabolism in the rat. *Amer J Physiol* 1965. 202. 883-886.
- WILSON, P. and P. A. R. WILSON, Changes in aortic glycosaminoglycans in rats fed hypercholesterolemic diet. *Atherosclerosis* 1971. 14. 63-77.
- MAX, S. R., P. DOLAN, R. RADHAKRISHNAMOORTHY and G. S. BERENSON, Isolation of lipoprotein-lipid-mucopolysaccharide complexes from fatty streaks of human aorta. *Atherosclerosis* 1972. 16. 95-104.

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ECG-changes in the fetal lamb during asphyxia in relation to beta-adrenoceptor stimulation and blockade

By

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Abstract

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ECG-changes in the S-T interval of the fetal electrocardiogram (FECG) were studied in 14 lambs, mostly unanesthetized and subjected to graded hypoxia. The aims of the study were to investigate if beta-adrenoceptor stimulation and blockade exerted additive or potentiating effects on the S-T and several cardiovascular parameters and whether the hypoxic changes of the FECG could be modified by beta-adrenoceptor blocking agents. The FECG changes were studied in order to correlate them with cardiovascular function, as measured by heart rate, mean arterial pressure, end diastolic pressure, stroke output and combined cardiac output, estimated by the thermodilution method, as well as with arterial pH, venous and base excess, blood lactate and glycerol. Intravenous doses of 0.02 to 0.4 $\mu\text{g kg}^{-1} \text{ min}^{-1}$ of isoprenaline induced the same pattern of changes in the FECG as we have previously recorded during asphyxia. By increasing the isoprenaline dose an increase in the duration of the FECG changes and a decrease in the T-wave changes was obtained. Propranolol was found to completely abolish the FECG changes induced by isoprenaline, as well as by mild hypoxia. During severe hypoxia the FECG changes were not abolished by propranolol. Our previous findings indicated that the hypoxic changes could be related to a sign of myocardial glycolysis. Thus, the present finding that even small doses of isoprenaline modified the S-T interval, because the same pattern of FECG changes corroborates this hypothesis.

The fetal electrocardiogram (FECG) is used in clinical practice mainly as a means to obtain an assessment of fetal heart rate (FHR). While it is generally accepted that FHR is an indicator of fetal well-being during pregnancy and labour the diagnostic value of the FECG is controversial. Although alterations in the pattern of the FECG have been detected during labour (Davidson 1971, Pardi *et al.* 1974) by using the fetal scalp electrode, these alterations are believed to be preceded by changes in the FHR (Hon and Lee 1963, Leo and Hon 1965, Davidson 1971).

Several investigators have shown changes in the FECG during hypoxia using experimental animals (Enbom and Westin 1954, Stern *et al.* 1961, Geill and Gyulai 1969, Mueller and Schach *et al.* 1971, Myers 1972, Morishima *et al.* 1975). Although changes in the S-T interval were readily apparent, it was stated that the use of FECG for the detection of fetal hypoxia will be limited.

- SVANBORG, A. and L. SVENNERHOLM, Plasma total lipid, cholesterol, triglycerides, phospholipids and fatty acids in a healthy scandinavian population. *Acta med. scand* 1961 169 43-49.
- SVENNERHOLM, L., Distribution and fatty acid composition of phosphoglycerides in normal human. *J. Lipid Res* 1968, 9 570-579
- THUNELL, S., Procedures for the microscale investigation of vessel wall glycosaminoglycans. *Lab. Invest* 1967, Section II No. 9
- TUCKER, M. J. The effect of clofibrate on spontaneous arteriosclerosis in rats. *Atherosclerosis* 1971 255-263
- VIKARI, J. and H. SAARNI, Faster lipoprotein isolation by gradient flotation. *Chin. Chem. Lett.* 1973
- VUOLAKKUMAR, S. T. S. LEYLAMMA and P. A. KURUP, Changes in aortic glycosaminoglycan and protein lipase activity in rats with age and atheroma. *Atherosclerosis* 1975, 21 1-14
- WOESSNER, J. F. JR., The determination of hydroxyproline in tissue and protein samples containing small proportions of this amino acid. *Arch. Biochem. Biophys.* 1961 93 440-447

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ECG-changes in the S-T interval of the fetal electrocardiogram (FECG) were studied in 14 lambs
anesthetized and subjected to graded hypoxia. The aims of the study were to investigate
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beta-adrenoceptor blockade on the S-T interval and whether the hypoxic changes of the FECG could be
abolished by beta-adrenoceptor blocking agents. The FECG changes were studied in order to correlate them
with cardiovascular function, as measured by heart rate, mean arterial pressure, and diastolic pressure,
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pH, base excess, blood lactate and glucose. Injections of small doses (0.02 to 0.4 µg kg⁻¹ min⁻¹)
of isoprenaline induced the same pattern of changes in the FECG as we have previously recorded during
hypoxia. By increasing the isoprenaline dose an increase in the duration of the FECG changes and
the T-wave changes was obtained. Propranolol was found to completely abolish the FECG
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interval were readily apparent, it was stated that the use of FECG for the detection of fetal
hypoxia will be limited.

Our own animal experiments revealed progressive and reproducible changes in the interval of the FECG during graded hypoxia both in mature guinea-pig fetuses and in fetuses (Rosén and Kjellmer 1975). The metabolic background of these changes of FECG was studied from different aspects. Rosén and Isaksson (1976) demonstrate parallelism between the FECG changes and the depletion of myocardial glycogen during graded hypoxia in guinea pig fetuses. The significance of this finding was supported by the relationship found between the increase in the amplitude of the T wave and the degree of metabolic acidosis as well as the accumulation of lactate in the exteriorized fetal heart (Rosén *et al.* 1976). These findings together with the relationship found between the appearance of the FECG-changes and decrease in PaO₂ (Rosén, Hökegård and Kjellmer 1975) indicate that the progressive FECG changes reflect myocardial glycogenolysis. Alterations in the FECG pattern consistently preceded signs of failing cardiovascular function. These occurred well in advance of any bradycardia (Rosén, Hökegård and Kjellmer 1975).

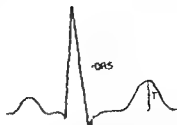
Thus, there are a good many observations indicating that progressive changes in the interval of the FECG reflect myocardial glycogenolysis and early hypoxic stress. The augmented glycogenolysis in heart muscle by beta-receptor stimulation is well known (Mayer 1967). Our own preliminary experiments indicated that the beta-receptor stimulant isoprenaline, initiated the same typical pattern of changes in the FECG as hypoxia. Therefore it was considered of interest to elucidate the influence of beta-adrenergic stimulation and blockade on the FECG.

The aims of the present study were to investigate whether beta-adrenoceptor stimulation and hypoxia exerted additive or potentiating effects in the FECG and several cardiovascular parameters and whether the hypoxic changes of the FECG could be blocked by beta-adrenoceptor blocking agents.

Material and methods

The experiments were conducted on 9 ewes of mixed breed with 14 fetuses acutely exteriorized. Gestational age was dated in 2 ewes and was estimated from fetal weight and crown-rump length using standard curves (Joubert 1956). In a first series of 5 ewes with 8 fetuses, the experiments were conducted according to the methods described earlier (Rosén, Hökegård and Kjellmer 1976). Their gestational age ranged from 109 to 132 days (term 147 days) and their weight from 1 060 to 3 370 g (2 441 ± 170 mean value ± S.E.). The left jugular vein of the fetus was cannulated and 0.1–10.0 µg (0.04 to 0.83 mg/kg) isoprenaline was given by single injections. Via the same catheter the beta-blocking agent, propranolol, was given in doses varying from 0.05 to 2 mg (0.02 to 0.83 mg/kg). Fetal and maternal blood pH and blood gas tensions were measured, fetal heart rate and end diastolic pressure were recorded. Combined fetal cardiac output was estimated and the FECG recorded with CR-lead. The ewes and thereby the fetuses were exposed to alternate periods of normoxia and hypoxia. The hypoxia was induced by ventilating the ewe with gas mixtures containing 9–15% O₂ in N₂ and in some cases with an addition of 10–20% CO₂. The hypoxic changes in the ECG pattern were quantified according to the scoring system previously described.

- Grade I The appearance of negative T wave changes, the amplitude exceeding that of the P wave.
 Grade II Maximally negative T wave changes.
 Grade III A gradual decrease in the amplitude of the negative T wave changes.
 Grade IV An elevation of the S-T segment and the T wave, the amplitude of the T wave being less than that of the P wave.
 Grade V A maximal increase in the amplitude of the T wave.
 Grade VI A decrease in the amplitude of the T wave during continuous hypoxia.



Example of the calculation of the T/QRS ratio.

first series of 4 ewes with 6 fetuses was used to establish quantitative dose-response relationship between isoprenaline and FECG changes. Their gestational age ranged from 128 to 133 days (132 ± 2 , mean \pm S.E.) and their weight from 2.330 to 2.930 g (2.530 ± 100). Maternal and fetal blood pressure, blood gases and pH, fetal heart rate and FECG are registered in the same way as in the first series, or to get quantitative measurement of the high and peaked T waves, ratio between the amplitude of QRS complex and the T wave (T/QRS ratio) was calculated (Fig. 1).

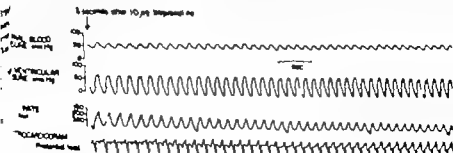
Fetal pressure was monitored with micro-tip sensitive catheter (Miller Instruments), inserted into the left carotid artery. Maximum dP/dt was derived electronically from the signal and continuously recorded. The thermocatheter method (Hindvall *et al.* 1973) was also used for estimation of the fetal cardiac output ("CO"), but instead of injecting saline of room temperature into the left atria as in the first series, it was injected into the inferior vena cava through catheter inserted in the right vein. The left jugular vein was cannulated and isoprenaline was administered continuously with an infusion pump in stepwise higher doses of 0.044, 0.076, 0.144, 0.22, 0.79 and 4.4 μ g/min in order to get dose-response curve. This was done both during normoxia and hypoxia before and after beta-sympathetic block. The beta-blocking agent propranolol was given through the same catheter in the right vein at doses varying from 0.02 to 0.5 mg (0.01 to 0.19 mg/kg).

Results

Beta-sympathetic stimulation

In the first series, isoprenaline was given i.v. in single doses ranging from 0.04 to 9.0 μ g/kg fetuses. In each case isoprenaline induced the same alterations of the FECG pattern as in the first series.

A relationship was found between the dose of isoprenaline and the increase in T wave amplitude, as well as the duration of the ECG changes. Fig. 2 gives an example of changes made after the injection of 1.0 μ g (0.4 μ g/kg) isoprenaline. The whole sequence of alterations of the S-T interval, accounted for in the ECG-scoring system is demonstrated.



2. Fetal electrocardiogram changes after the injection of 1.0 μ g (0.4 μ g/kg) isoprenaline. Note the rapid increase in the S-T interval.

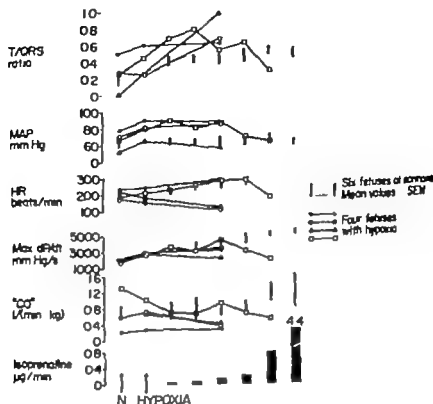


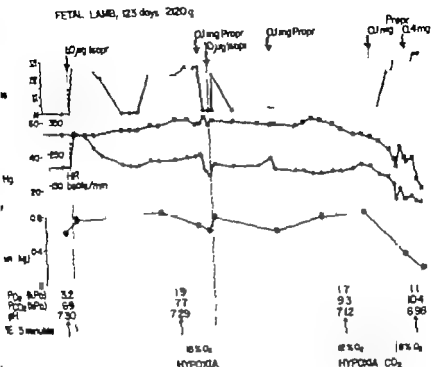
Fig. 3 Effect of isoprenaline infusion on the T/QRS ratio of the fetal electrocardiogram, fetal heart rate, mean arterial pressure, maximum dP/dt and combined cardiac output both during normoxia and hypoxia.

In order to further elucidate the influence of beta-adrenoceptor stimulation on the FHR and cardiovascular function and to estimate a dose response curve, isoprenaline was continuously infused in step-wise higher doses in a second series. This group of 6 fetuses was quite homogeneous regarding gestational age and fetal weight.

ECG-changes Fig. 3 demonstrates the relationship between the increase in T wave amplitude and the dose of isoprenaline (6 fetuses). Hypoxia (4 fetuses) had an inhibitory effect on the ECG changes which appeared earlier and with a higher T/QRS ratio (Grade V in the scoring system). At the highest dose of isoprenaline given during hypoxia the T/QRS ratio decreased again (Grade VI in the scoring system) in the only animal studied.

Cardiovascular function Fig. 3 also demonstrates the effect of isoprenaline on mean arterial pressure, fetal heart rate, maximum dP/dt and combined cardiac output. Both FHR, dP/dt and cardiac output increase gradually with increasing doses of isoprenaline. MAP shows no significant changes. The augmentation of cardiac performance caused by isoprenaline infusion was inhibited by the combination of hypoxia and acidosis. Fig. 3 demonstrates the combined effect of hypoxia and isoprenaline in 4 fetuses. After an initial increase associated with hypoxia both MAP, FHR, maximum dP/dt and "CO" decrease at increasing doses of isoprenaline.

Blood gases, pH, lactate and glucose There was no change in PaO₂ during the isoprenaline infusions at normoxia. PaO₂ was 3.19 ± 0.29 kPa before the infusions started and 3.09 ± 0.24 kPa during the infusions.



4 Changes in the fetal electrocardiogram, graded according to the scoring system, mean arterial pressure, fetal heart rate and combined cardiac output after isoprenaline and propranolol injections made during normoxia, hypoxia and the combination of hypoxia and hypercapnia.

At the end of the infusions (mean value \pm S.E.), pH, however, decreased from 7.27 ± 0.04 to 7.17 ± 0.06 and there was a lactate accumulation from 2.85 ± 0.47 to 4.77 ± 1.69 mmol/l. Blood glucose values did not change (1.47 ± 0.28 and 1.53 ± 0.30 mmol/l respectively). In the 4 fetuses subjected to both hypoxia and isoprenaline infusion the mean PaO₂ was 3.53 (range 3.19–3.86) before hypoxia and decreased to 1.86 (range 1.06–2.66) during hypoxia and 1.83 (range 1.06–2.52) when isoprenaline infusion was added. The mean pH value was 7.15 (range 6.99–7.24) before hypoxia and decreased to 7.04 (range 6.89–7.18) during hypoxia and isoprenaline infusion. The mean lactate value was 5.94 (range 1.71–7.22) before the hypoxic period and the concentration rose to 8.58 (range 1.71–13.16) mmol/l during hypoxia and isoprenaline infusion. Blood glucose values increased slightly from 1.96 (range 1.74–2.20) to 2.20 (range 2.07–2.36) mmol/l. In all these 4 fetuses the period of hypoxia combined with isoprenaline was preceded by periods of isoprenaline infusion without hypoxia.

α -adrenoceptor blockade

Propranolol was also given to the 8 fetuses of the first series in order to test whether β -adrenoceptor blockade interfered with the ECG changes induced by hypoxia. The degree of

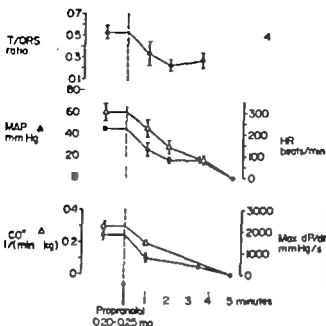


Fig. 5. Effect of propranolol on fetal cardiovascular function. The fetus was subjected to hypoxia and acidosis. The arterial blood pressure was 2.21 ± 0.24 kPa before the injection and 1.97 ± 0.39 after the injection. The heart rate was 6.98 ± 0.13 before the injection and 0.09 after the injection (mean \pm S.E.).

beta blockade was tested by repeated injections of isoprenaline. The ECG changes elicited by isoprenaline completely disappeared about 10 s after the propranolol injections.

Fig. 4 demonstrates the pattern of events recorded in a lamb fetus (gestational age 123 days). The first part of the figure illustrates the effect of isoprenaline ($0.47 \mu\text{g/kg}$) on combined cardiac output, heart rate and mean arterial pressure as well as the fetal ECG. Alterations in the FECG pattern: elevation of the S-T segment with high and peaked T waves, were associated with a tachycardia and an increase in cardiac output from 0.78 l/(min kg). A moderate hypoxia, induced by ventilating the ewe with $15\% \text{O}_2$, elicited the same pattern of events in the FECG. These changes were immediately blocked by propranolol (0.05 mg/kg). This dose of propranolol was found to almost completely block the effect of a large dose of isoprenaline ($4.7 \mu\text{g/kg}$). In the last part of the figure the effects of beta blockade associated with a severe hypoxia are demonstrated. No FECG changes were recorded until there were signs of falling cardiovascular function. These FECG changes could apparently not be blocked even by large doses of propranolol. Propranolol blocked the FECG changes elicited by moderate hypoxia in the two fetuses studied but did not block the FECG changes associated with a severe hypoxia and a failing circulation (6 cases). One fetus was extremely susceptible to the combination of hypoxia and propranolol.

In the second series propranolol blocked the cardiovascular effects of isoprenaline in the same way. These fetuses were also extremely susceptible to the combination of propranolol and hypoxia. In 4 fetuses the combination of acidosis, hypoxia and propranolol resulted in rapid deterioration of fetal circulation and fetal death 3 to 8 min after the injection of 0.2 to 0.25 mg propranolol i.v. Fig. 5 illustrates the rapid decay of MAP, FHR, maximal dP/dt and "CO". There is also a decrease in the amplitude of the maximally high peaked T waves during hypoxia after propranolol injection, i.e. grade VI in the ECG system.

Discussion

results demonstrate that small doses of isoprenaline given during normoxia can elicit the sequence of events in the FECG previously recorded during hypoxia. This is in accordance with the view that glycogenolysis induced by beta-adrenoceptor stimulation *in vivo* will produce the changes of the FECG. Most tissues respond to oxygen lack by increasing their rate of glycolysis, in parallel with the metabolic events elicited by beta-tor stimulation (Mayer *et al.* 1967). In asphyxiated animals adrenaline and noradrenaline may be released from the adrenal medulla leading to an increased rate of glycogen breakdown due to phosphorylase activation. This mechanism has been shown to operate *in vivo* (Corrofine and Silver 1965). Jones and Robinson (1975) measured increased plasma catecholamine concentrations during hypoxia (fetal P_{aO_2} 18 mmHg) in the chronically catheterized fetal lamb. They could demonstrate an increase in plasma adrenaline responding to that produced by the infusion of $0.4 \mu\text{g kg}^{-1} \text{min}^{-1}$ adrenaline. Thus, it can be seen that the dose of isoprenaline (0.02 to $0.4 \mu\text{g kg}^{-1} \text{min}^{-1}$) which we found to elicit changes in the FECG pattern, is similar to the amounts of catecholamines secreted during hypoxia. Furthermore, an increase in the dose of isoprenaline resulted in an increase in duration and amplitude of the T-wave changes in parallel with the pattern of changes recorded with increasing degree of fetal hypoxic stress. Alterations in the FECG with an increase in P wave and decrease in T wave amplitude together with a shortened P-R interval, have been recorded during catecholamine infusion into fetal rhesus monkey by Adamsons (1971) using significantly higher dosages. Stern *et al.* also obtained higher T-waves after administration of adrenaline ($100 \mu\text{g i.m.}$) to newborn babies (1960) and to human infants (1961).

Isoprenaline gave a positive chronotropic and inotropic effect, the latter nicely demonstrated by an increase in myocardial contractility as measured by an increase in max dP/dt . There was a decrease in pH and an accumulation of blood lactate during isoprenaline infusions but no change in P_{aO_2} . These findings are compatible with an augmented glycolysis. Infusion of isoprenaline had an additive effect to hypoxia and acidosis with larger and more pronounced FECG changes and seemed to give a more rapid deterioration of fetal circulation.

The positive chronotropic and inotropic effects of isoprenaline, as well as the negative inotropic effect of propranolol, have previously been studied in lamb fetuses (Jochanson *et al.* 1972, van Petten and Wilkes 1970). In our study even small doses of propranolol (0.1 to 0.19 mg/kg) completely abolished the FECG changes, produced either by isoprenaline or mild hypoxia. A different pattern was seen during more severe hypoxia with signs of falling cardiovascular function. In this situation beta-blockade had no influence on the ECG changes, consistent with the fact that asphyxia *per se* is a stimulator of phosphorylase activity in the myocardium (Mayer *et al.* 1967). The fetus was extremely susceptible to the combination of propranolol and hypoxia. This is likely to be the result of propranolol blocking the most important compensatory mechanisms on the circulatory (Leaning *et al.* 1969) as well as the metabolic side.

The question now to be asked is whether the predominant change in the FECG, i.e. the peaked T wave, is induced by the augmentation of myocardial glycolysis: a direct effect of the high catecholamine output on the function of the myocardial membrane. There are a number of findings in support of the former hypothesis: *A* Even during the infusion of very high doses of isoprenaline during normoxia no T wave changes were recorded. *B* Hypoxia had a marked additive effect on the high T waves indicating a mechanism other than beta-adrenoceptor stimulation: for example a direct stimulation of phosphorylase activity in the myocardium. *C* The propranolol rapidly abolished the effect of isoprenaline, as well as the ECG changes induced by mild hypoxia, no such response was seen on the ECG during earlier hypoxia further indicating another mechanism than beta-stimulation behind the ECG changes. During isoprenaline infusion there was an increase in lactate concentration which is of cardiac origin (Dawes *et al.* 1959).

The conclusion to be made is that mild hypoxia initiates the ECG changes: activation of beta-adrenoceptors while severe hypoxia induces the ECG changes by a direct effect.

Thus, progressive changes in the S-T segment of the FECG during hypoxia reflect myocardial glycolysis induced by an imbalance between energy-yielding and energy-consuming processes.

This study was supported by grants from Göteborgs Läkaresällskap, the Faculty of Medicine, Göteborg, and the Swedish Medical Research Council (2591) and Prenatalforskningsfonden.

References

- ADAMSON, K., E. MUELLER HEUBACH and R. E. MYERS, Production of fetal asphyxia in the monkey by administration of catecholamines to the mother. *Amer. J. Obstet. Gynec.* 1971 109 78.
- COMLINE, R. S., I. A. SILVER and M. SILVER, Factors responsible for the stimulation of the adrenal medulla in the fetal lamb. *J. Physiol. (Lond.)* 1965 178 11-23.
- DAWES, G. S., J. C. MOTT and H. J. SHELLEY, The importance of cardiac glycogen for the newborn life in foetal lambs and newborn animals during anoxia. *J. Physiol. (Lond.)* 1959 106 514-532.
- DAVIDSEN, P. C. B., The significance of the foetal electrocardiogram during labour with detailed report on one case. *Acta obstet. gynec. scand.* 1971 50 43-49.
- DOWNING, S. E., T. H. G. RÖMER and J. H. ROCAMORA, Adrenergic support of cardiac function in hypoxia in the newborn lamb. *Amer. J. Physiol.* 1969 217 722-735.
- EACHUSON, G. and B. WESTIN, Experimental studies of the human fetus in prolonged asphyxia. *Physiol. scand.* 1954 31 359-375.
- GILL, M. G. and F. GYULAI, Effect of glucose infusion in the mother before delivery on the ECG of the foetuses under anoxia. *Acta obstet. gynec. scand.* 1969 48 56-63.
- HEDVALL, G., I. KJELLMER and T. OLSSON, A experimental evaluation of the thermodynamic method for determination of cardiac output and of intracardiac right to-left shunts. *Scand. J. Clin. Lab. Invest.* 1973 31 61-68.
- HOW, E. H. and S. T. LEE, The fetal electrocardiogram. I. The electrocardiogram of the human fetus. *Amer. J. Obstet. Gynec.* 1963 87 804-813.
- JÖNSSON, I., M. D. BARTON, S. DANIEL, L. S. J. MEYER and K. ADAMSON, The response of the maternal sheep fetus to sympathicomimetic amines and adrenergic blocking agents. *Amer. J. Obstet. Gynec.* 1972 114 43-50.
- JONES, C. T. and R. O. ROBINSON, Plasma catecholamines in foetal and adult sheep. *J. Physiol. (Lond.)* 1975 248 15-33.
- JOURET, D. M., A study of pre-natal growth and development in the sheep. *Sci.* 1963 47 30.

12. F. and E. H. HOLT, The fetal electrocardiogram. IV Unusual variations in the QRS complex during labor. *Amer J Obstet Gynec.* 1963. 92. 1140-1148.
13. S. E. B. J. WILLIAMS and J. M. SMITH, Adrenergic mechanisms in cardiac glycogen metabolism. *N Y Acad. Sci.* 1967. 139. 686-702.
14. J. H. H. S. S. DAVIES, R. T. RICHARDS and L. S. JAMES, The effect of increased maternal P_{aO_2} on the fetus during labor. *Amer J Obstet. Gynec.* 1975. 123. 257-264.
15. S. HEURACHE, E. and K. ADAMS, Surveillance of the fetus during the intrapartum period. *Am. J. Med.* 1971. 39. 427-439.
16. R. E., Two patterns of perinatal brain damage and their conditions of occurrence. *Amer J Obstet.* 1972. 112. 246-276.
17. G. E. TUCCI, A. UNDERO and D. ZANFI, Fetal electrocardiogram changes in relation to fetal rate patterns during labor. *Amer J Obstet. Gynec.* 1974. 118. 243-250.
18. TITZ, G. R. and R. F. WELLS, Beta-adrenergic responses in the unanesthetized ovine foetus. *J Pharmacol.* 1970. 39. 572-582.
19. K. O. and I. KELLMER, Changes in the fetal heart rate and ECG during hypoxia. *Acta physiol. scand.* 1974. 91. 39-66.
20. K. O. and O. HANSSON, Alterations in fetal heart rate and ECG correlated to glycogen, inosinophosphate and ATP levels during graded hypoxia. *Neonol. Neonata.* 1976. 30. 17-24.
21. K. O. K.-H. HÖRSTGÅRD and I. KELLMER, A study of the relationship between the electrocardiogram and hemodynamics in the fetal lamb during asphyxia. *Acta physiol. scand.* 1976. 98. 275-284.
22. K. O., A. HANSE, K. KARLSSON, I. KELLMER, T. OLSSON and M. RUMA, Changes in the ECG and its sensory evoked EEG responses (SER) during intrauterine asphyxia in the sheep. *Eur. Neurol.* 1977. 30. 95-101.
23. L. and J. LIND, Neonatal T wave patterns. *Acta paediat. scand.* 1960. 49. 329-337.
24. L. J. LIND and B. KAPLAN, Direct human foetal electrocardiography (with studies of the effect of nitrite, streptoz, clamping of the umbilical cord and placental separation on the foetal ECG). *Neonol. (Basel)* 1961. 3. 49-62.

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References

- ADAMSON, K., E. MUELLE HEUBACH and R. E. MYERS, Production of fetal asphyxia in the monkey by administration of catecholamines to the mother. *Amer. J. Obstet. Gynec.* 1971 **100** 94.
- COMLINE, R. S., J. A. SILVER and M. SILVER, Factors responsible for the stimulation of the adrenal during asphyxia in the fetal lamb. *J. Physiol. (Lond.)* 1965 **178** 211-238.
- DAWES, G. S., J. C. MOTT and H. J. SHELLEY, The importance of cardiac glycolysis for the maintenance of life in foetal lambs and newborn animals during anoxia. *J. Physiol. (Lond.)* 1959 **146** 516-533.
- DAVIDSEN, P. C. B., The significance of the foetal electrocardiogram during labour with detailed case. *Acta obstet. gynec. scand.* 1971 **50** 45-49.
- DOWNING, S. E., T. H. GARDNER and J. M. ROCAMORA, Adrenergic support of cardiac function in hypoxia in the newborn lamb. *Amer. J. Physiol.* 1969 **217** 728-735.
- ENHÖRNING, H. and B. WESTIN, Experimental studies of the human fetus in prolonged asphyxia. *Acta obstet. gynec. scand.* 1954 **31** 359-375.
- GILLI, M. G. and F. GYULAI, Effect of glucose infusion in the mother before delivery on the ECG of the foetuses under anoxia. *Acta obstet. gynec. scand.* 1969 **48** 56-63.
- HEDVALL, G., J. KJELLMER and T. OLSSON, An experimental evaluation of the thermodynamic method for determination of cardiac output and of intracardiac right-to-left shunts. *Scand. J. Clin. Lab. Invest.* 1973 **31** 61-68.
- HON, E. H. and S. T. LEE, The fetal electrocardiogram. I. The electrocardiogram of the dysmature fetus. *Amer. J. Obstet. Gynec.* 1963 **87** 804-813.
- JOHANSSON, I., M. D. BARTON, S. DANIEL, L. S. JAMES and K. ADAMSON, The response of the neonatal sheep fetus to sympathicomimetic amines and adrenergic blocking agents. *Amer. J. Obstet. Gynec.* 1972 **114** 43-50.
- JONES, C. T. and R. O. ROBINSON, Plasma catecholamines in foetal and adult sheep. *J. Physiol. (Lond.)* 1975 **248** 15-33.
- JOUBERT, D. M., A study of pre-natal growth and development in the sheep. *J. Agr. Sci.* 1963 **47** 30.

- and E. H. HON, The fetal electrocardiogram. IV. Unusual variations in the QRS complex during labor. *Amer. J. Obstet. Gynec.* 1963. 92, 1140-1148.
- S. E., B. J. WILLIAMS and J. M. SMITH, Adrenergic mechanisms in cardiac glycolysis metabolism. *N. Y. Acad. Sci.* 1967. 139, 686-702.
- MA, H. O., S. DANIEL, R. T. RICHARDS and L. B. JAMES, The effect of increased maternal PaO_2 on the fetus during labor. *Amer. J. Obstet. Gynec.* 1973. 123, 257-264.
- HRUBCZAK, E. and K. ADAMSON, Surveillance of the fetus during the intrapartum period. *M. J. Aust.* 1971. 80, 427-439.
- R. E. Two patterns of perinatal brain damage and their conditions of occurrence. *Amer. J. Obstet.* 1972. 112, 246-276.
- G., E. TUCCI, A. UDRIZO and D. ZAMPA, Fetal electrocardiogram changes in relation to fetal rate patterns during labor. *Amer. J. Obstet. Gynec.* 1974. 118, 243-250.
- TEV, G. R. and R. F. WELLS, Beta-adrenergic responses to the unanesthetized ovine fetus. *J. Pharmacol.* 1970. 38, 572-582.
- K. O. and I. KJELLMER, Changes in the fetal heart rate and ECG during hypoxia. *Acta physiol. scand.* 1971. 93, 59-66.
- K. O. and O. HANSSON, Alterations in fetal heart rate and ECG correlated to glycogen, inorganic phosphate and ATP levels during graded hypoxia. *Neonate.* 1976. 30, 17-24.
- K. O. K.-H. HÖKRELLER and I. KJELLMER, A study of the relationship between the electrocardiogram and hemodynamics in the fetal lamb during asphyxia. *Acta physiol. scand.* 1976. 93, 275-284.
- K. O., A. HENRIK, K. KALLSTRÖM, I. KJELLMER, T. OLSSON and M. ROSS, Changes in the ECG and to-tensity evoked EEO responses (SER) during intrauterine asphyxia in the sheep. *Neonate.* 1976. 30, 93-101.
- L. and J. LIND, Neonatal T wave patterns. *Acta paediatr. scand.* 1960. 49, 329-337.
- L. J. LIND and B. KAPLAN, Direct human fetal electrocardiography (1st studies of the effect of saline, atropine, clamping of the umbilical cord and placental separation on the fetal ECG). *Konink. (Soc.)* 1961. 3, 49-62.

Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man

By

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Abstract

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To investigate the effects of sustained positive-pressure breathing (PPB) on the adaptation of respiratory and circulatory functions to exercise 8 healthy volunteers were exposed to PPB of air at 15 and 30 cm H₂O in the supine position at rest and while performing leg exercise at 50% of individual maximal capacity. PPB was both subjectively and objectively better tolerated when combined with exercise than when it was at rest. PPB at 30 cm H₂O resulted in marked hyperventilation with alkalosis in the resting state but did not significantly affect respiratory minute volume, blood gases or acid-base balance during exercise. Cardiac output and left ventricular work were reduced by about one fifth and one third, respectively, at rest and during exercise. In contrast to the case at normal airway pressure, exercise-induced increase in cardiac output was accompanied by a decrement in stroke volume during PPB. Although mean airway pressure (relative to atmospheric) was elevated by PPB at rest and during exercise the driving pressure in the systemic circuit (arterial minus central venous pressure) was reduced in both conditions. It is concluded that dynamic exercise counteracts deleterious effects of PPB by normalizing respiratory function and improving cardiac filling by activation of the leg muscle and the abdominal pumps.

Key words. Airway pressure, exercise, cardiac output, baroreflexes, arterial pressure regulation

Numerous observations are available in the literature concerning the effects of constant positive pressure in the airways on circulatory and respiratory functions in the resting condition. Most of these refer to the anesthetized animal (for review see Lenfant and Hargens 1960). Physiologic consequences in resting, conscious man which in important ways differ qualitatively from those in the anesthetized state, have been reviewed by Luft (1967). Continuous positive-pressure breathing (PPB) depresses cardiac output due to obstruction to venous inflow into the abdomen and chest, blood thus being displaced into peripheral distensible veins outside the trunk.

PPB is a powerful tool for the study of compensatory mechanisms that act to maintain homeostasis against loss of effective circulating blood volume. We are not aware of

tion is available on the combined effects of PPB and dynamic exercise. In this situation cardiac function is taxed by increased demands on the circulation and at the same time mechanically interfered with by the increased pressure in the airways. The present experiments were designed to study the influence of PPB on the adaptation of pulmonary and vascular functions to moderate, dynamic leg exercise in supine man.

Methods

and experimental setup. 8 healthy male volunteers, aged 23-37 years, participated as test subjects. Informed consent was obtained. Mean body weight was 78.0 (range 65-87) kg, and mean \dot{V}_{O_2} max was 3.0 (range 2.0-3.5) $\text{lit kg}^{-1} \text{ min}^{-1}$ as determined from nomograms of Astrand (1960). In all experiments, 4 ways of the supine subjects were connected via mouth-piece, respiratory valve and large-calibre tracheal and expiratory tubes to a ventilated pressure chamber 35 m^3 of volume. The inside pressure in the chamber was controlled to give the desired mean airway pressure (0, +15 and +30 cm H_2O) within ± 1 cm H_2O . Tidal volume, and respiratory rate were recorded continuously on-line on breath-by-breath (Astrand and Wigertz 1964), using signals from Fleisch flow-meter incorporated on the inspiratory side of the respiratory valve. Pressure in the mouth-piece was monitored and recorded continuously on sensitive pressure transducers by the inspiratory and expiratory conduits connected to 10 and 0.6 cm^2 at flow rate of 1.0 l min^{-1} and to 17 and 13 cm H_2O at flow rate of 2.0 l min^{-1} respectively. The heart rate was recorded from chest electrodes by means of a low-resistance, beat-to-beat heart-rate meter (Lindholm and Wigertz 1969). Intrathoracic pressure was recorded from Teflon catheter in radial artery. Mean arterial pressure was derived electronically. The central venous pressure was recorded via catheter introduced via cubital vein with its tip in or near the right atrium. Cardiac output was measured by the dye-dilution method (indocyanine green, Cardio-Green[®]) as described elsewhere (Bjorklund and Tykka 1977), using the central venous catheter for dye injections. Central blood flow was calculated from the dye-dilution curves in accordance with the Stewart-Hamilton formula. Acid-base balance was measured with calibrated electrodes at 37°C (Instrumentation Laboratory or Radiometer) in spot-samples drawn from the radial-artery catheter. All signals were displayed on a mechanical strip-chart recorder (Brush Mk 200) and on magnetic tape (Ampex FR 2200) for storage and subsequent detailed analysis of the variables during the study. In addition, the dye-dilution curves were inscribed on an X-Y recorder (Hewlett-Packard 404).

Experimental procedure. The experiment was conducted with the subject in the supine position on a bed, as provided with fixed shoulder support to avoid isometric work with the arms as the subject lay on his back. An electrically stabilized cycle ergometer (Eliason-Schneider) was attached to the bed frame in such a way that the subject kept his feet on the pedals also in the resting condition. In order to avoid any isometric work, subjects were thoroughly familiarized with the subjective sensations of leg exercise combined with PPB at the pressure levels to be used subsequently in the experimental study. In one of these sessions the individual aerobic power was determined using the nomograms of Astrand (1960). The experimental protocol (Fig. 1) comprised 3 parts. In the first, the responses to PPB were studied with the subject resting, with 4 min exposure to PPB at +15 and +30 cm H_2O . In the 2nd part of the protocol, separated by a 45 min recovery period, the subject exercised for 15 min at a constant rate of leg exercise, with and without PPB at +15 and +30 cm H_2O . The sequence of the two parts was rotated among the subjects. The exercise run without PPB served to give individual data on spontaneous base-line shifts of the variables under study occurring during 'steady-state' exercise. The 6th through the 12th min. By compensating for these base-line shifts it was possible to study the changes produced by PPB during exercise. The data reported here refer to the last min in each condition (cf. Fig. 1).

Calculations. Heart rate and mean arterial pressure were averaged during the inscription of each indicator curve. Total peripheral resistance was calculated as the difference between mean arterial and mean central venous pressures (mmHg) divided by cardiac output (ml/min), left ventricular work (watt) as the product of cardiac output (l/min) and the difference between mean arterial and mean central venous pressures (mmHg), divided by 450.1. The statistical significance of differences between mean values was assessed by applying the *t*-test to the inter-individual differences (cf. Fisher 1948).

Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man

By

H BJURSTEDT, G ROSENHAMER, B LINDBORG and C. M. HESSER

Received 1 June 1978

Abstract

BJURSTEDT H, G ROSENHAMER, B LINDBORG and C. M. HESSER. *Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man.* Acta physiol. scand. 1979 105: 204-214.

To investigate the effects of sustained positive-pressure breathing (PPB) on the adaptation of respiratory and circulatory functions to exercise 8 healthy volunteers were exposed to PPB of air at 13 and 18 cm H₂O in the supine position at rest and while performing leg exercise at 50% of individual maximal capacity. PPB was both subjectively and objectively better tolerated when combined with exercise than at rest. PPB at 30 cm H₂O resulted in marked hyperventilation with alkalosis but did not significantly affect respiratory minute volume, blood gases or acid-base balance. Cardiac output and left ventricular work were reduced by about one fifth and one third, respectively, at rest and during exercise. In contrast to the case at normal airway pressure, exercise-induced increase in cardiac output was accompanied by an increment in stroke volume during PPB. Although mean arterial pressure (relative to atmospheric) was elevated by PPB at rest and during exercise, the driving pressure in systemic circuits (arterial minus central venous pressure) was reduced in both conditions. It is concluded that dynamic exercise counteracts deleterious effects of PPB by normalizing respiratory function and improving cardiac filling by activation of the leg muscle and the abdominal pump.

Key words: Airway pressure, exercise, cardiac output, baroreflexes, arterial pressure regulation.

Numerous observations are available in the literature concerning the effects of constant positive pressure in the airways on circulatory and respiratory functions in the awake condition. Most of these refer to the anesthetized animal (for review see Lenfant and Hlastala 1960). Physiologic consequences in resting, conscious man, which in important aspects differ qualitatively from those in the anesthetized state, have been reviewed by Luft (1972). Continuous positive-pressure breathing (PPB) depresses cardiac output due to obstruction to venous inflow into the abdomen and chest, blood thus being displaced peripherally into distensible veins outside the trunk.

PPB is a powerful tool for the study of compensatory mechanisms that act to maintain homeostasis against loss of effective circulating blood volume. We are not aware of

tion is available on the combined effects of PPB and dynamic exercise. In this situation cardiac function is taxed by increased demands on the circulation and at the same time mechanically interfered with by the increased pressure in the airways. The present experiments were designed to study the influence of PPB on the adaptation of pulmonary and vascular functions to moderate, dynamic leg exercise in supine man.

Methods

and experimental setup. 8 healthy male volunteers, aged 23-37 years, participated as test subjects. Various consent was obtained. Mean body weight was 78.0 (range 65-87) kg, and mean $\dot{V}O_{2\max}$ was 3.0 (range 2.0-3.5) $\text{l} \cdot \text{min}^{-1}$ as determined from nomogram of Astrand (1960). In all experiments, lungs of the subject were connected via mouth-piece, respiratory valve and large-calibre inspiratory and expiratory tubes to a ventilated pressure chamber, 33 m^3 of volume. The inside pressure was controlled to give the desired mean airway pressure (0, +15, and +30 cm H_2O) within ± 1 cm H_2O . Tidal volume, and respiratory rate were recorded continuously on-line on breath-by-breath (Larsson and Wigertz 1960), using signals from Fleisch flow-meter interposed on the inspiratory line of the respiratory valve. Pressure in the mouth-piece was monitored and recorded continuously on-line on the pressure offered by the inspiratory and expiratory conduits connected to 1.0 and 0.6 $\text{cm}^3 \cdot \text{s}^{-1}$ flow rates of 1.0 $\text{l} \cdot \text{min}^{-1}$ and to 1.7 and 1.3 cm H_2O at flow rates of 2.0 $\text{l} \cdot \text{min}^{-1}$ respectively. Heart rate was recorded from chest electrodes by means of a linear, beat-to-beat heart-rate meter (Liedholm and Wigertz 1968). Intracranial pressure was recorded from Teflon catheter in radial artery. Arterial pressure was derived electronically. The central venous pressure was recorded via catheter introduced via cubital vein with its tip in or near the right atrium. Cardiac output was measured by the dye-dilution method (indocyanine green, Cardio-Overs[®]) as described elsewhere (Rybo, Rosenhamer and Tydén 1977), using the central venous catheter for dye injections. Central blood flow was calculated from the dye-dilution curves in accordance with the Saw-ari-Hassall formula. Blood gases and acid-base balance were measured with calibrated electrodes at 37°C (Boehringer-Mannheim or Radiometer) in spot-samples drawn from the radial-artery catheter.

Analysis. All signals were displayed on multichannel strip-chart recorder (Brush Mk 200) and on magnetic tape (Ampex FR 2200) for storage and subsequent detailed analysis of the variables studied. In addition, the dye-dilution curves were recorded on an X-Y recorder (Hewlett-Packard 340).

Arrangement of experiment. The experiment was conducted with the subject in the supine position on a bed, with a fixed shoulder support to avoid isometric work with the arms as the subject lay. A electrically stabilized cycle ergometer (Elema-Schönmader) was attached to the bed frame with crank axis at the level of the bed. The subject kept his feet on the pedals also in the resting condition. Introductory amount, subjects were thoroughly familiarized with the subjective sensations of leg exercise combined with PPB at the pressure levels to be used subsequently in the experimental trial. In one of these sessions the individual aerobic power was determined using the nomogram of Astrand (1960). The experimental protocol (Fig. 1) comprised 3 parts. In the first, the responses to PPB at rest with the subject resting, with 4 test exposures to PPB at +15 and +30 cm H_2O . In the 2nd part of the protocol, separated by 45 min recovery period, the subject exercised for 13 min at rest. In the 3rd part, the subject exercised for 13 min at rest with and without PPB at +15 and +30 cm H_2O . The sequence of the two parts was repeated among the subjects. The exercise run without PPB served to give individual data on spontaneous base-line shifts of the variables under study occurring during 'steady-state' exercise. The 4th through the 13th min. By compensating for these base-line shifts it was possible to study the changes produced by PPB during exercise. The data reported here refer to the last min. in each condition (Fig. 1).

Calculations. Heart rate and mean arterial pressure were averaged during the inscription of each indicator on curve. Total peripheral resistance was calculated as the difference between mean arterial and venous pressure (mmHg) divided by cardiac output (ml/min), left ventricular work (watt) as the product of cardiac output (ml/min) and the difference between mean arterial and mean central venous pressure (mmHg), divided by 450 l. The statistical significance of differences between mean values were tested by applying the *t*-test to the intrasubject differences (cf. Fisher 1948).

Respiratory and circulatory responses to sustained positive-pressure breathing and exercise in man

By

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To investigate the effects of sustained positive-pressure breathing (PPB) on the adaptation of respiratory and circulatory functions to exercise, 8 healthy volunteers were exposed to PPB of air at 15 and 30 cm H₂O in the supine position at rest and while performing leg exercise at 50% of individual maximal capacity. PPB was both subjectively and objectively better tolerated when combined with exercise than at rest. PPB at 30 cm H₂O resulted in marked hyperventilation with alkalosis in the resting condition but did not significantly affect respiratory minute volume, blood gases or acid-base balance during exercise. Cardiac output and left ventricular work were reduced by about one fifth and one third, respectively, at rest and during exercise. In contrast to the case with normal airway pressure, exercise-induced increase in cardiac output was accompanied by an increment in stroke volume during PPB. Although mean arterial pressure (relative to atmospheric) was elevated by PPB at rest and during exercise, the driving pressure in systemic circuits (arterial minus central venous pressure) was reduced in both conditions. It is concluded that dynamic exercise counteracts deleterious effects of PPB by normalizing respiratory function and improving cardiac filling by activation of the leg muscle and the abdominal pumps.

Key words: Airway pressure, exercise, cardiac output, baroreflexes, arterial pressure regulation.

Numerous observations are available in the literature concerning the effects of constant positive pressure in the airways on circulatory and respiratory functions in the resting condition. Most of these refer to the anesthetized animal (for review see Lenfant and Rahn 1960). Physiologic consequences in resting, conscious man, which in many respects differ qualitatively from those in the anesthetized state, have been reviewed by Luft (1972). Continuous positive-pressure breathing (PPB) depresses cardiac output due to obstruction to venous inflow into the abdomen and chest, blood thus being displaced peripherally into distensible veins outside the trunk.

PPB is a powerful tool for the study of compensatory mechanisms that act to maintain homeostasis against loss of effective circulating blood volume. We are not aware of

tion is available on the combined effects of PPB and dynamic exercise. In this situation, the function is taxed by increased demands on the circulation and at the same time locally interfered with by the increased pressure in the airways. The present experiments were designed to study the influence of PPB on the adaptation of pulmonary and vascular functions to moderate, dynamic leg exercise in supine man.

Methods

Subjects and experimental setup. Eight healthy male volunteers, aged 23-37 years, participated in these experiments. Informed consent was obtained. Mean body weight was 78.0 (range 65-87) kg, and mean \dot{V}_{O_2} was 2.0 (range 1.5-2.5) $\text{l} \cdot \text{min}^{-1}$ as determined from a nomogram of Astrand (1960). In all experiments, trachea of the seven subjects were cannulated via a mouth-piece, respiratory valve and large-calibre tracheal and expiratory tubes to a ventilated pressure chamber 33 m^3 of volume. The inside pressure was controlled to give the desired mean airway pressure (0, +15, and +30 cm H_2O) within ± 1 cm. Tracheal, tidal volume, and respiratory rate were recorded continuously on-line on a breath-by-breath (Astrand and Wigertz 1966), using signals from a Fleisch flow-meter incorporated in the inspiratory line of the respiratory valve. Pressure in the mouth-piece was monitored and recorded continuously at positive pressures offered by the inspiratory and expiratory conduits associated to 1.0 and 0.6 $\text{l} \cdot \text{min}^{-1}$ flow rate of 1.0 $\text{l} \cdot \text{min}^{-1}$ and to 1.7 and 1.3 cm H_2O at a flow rate of 2.0 $\text{l} \cdot \text{min}^{-1}$ respectively. \dot{V}_{O_2} was recorded from chest electrodes by means of a linear beat-to-beat heart-rate meter (Lindholm and Wigertz 1969). Intrathoracic pressure was recorded from a Teflon catheter in radial aorta. Mean arterial pressure was derived electronically. The central venous pressure was recorded via catheters introduced via cubital vein into the right atrium. Cardiac output was used by the dye-dilution method (iodocyanine green, Cardio-Green[®]) as described elsewhere (Ripstein and Tykocnik 1977), using the central venous catheter for dye injections. Central blood flow was calculated from the dye-dilution curves in accordance with the Stewart-Hamilton formula. Arterial blood gases and end-tidal balance were measured with calibrated catheters at 37°C (Baird Medical Laboratory or Radiometer) in spot-samples drawn from the radial-artery catheter during exercise. All signals were displayed on a multichannel strip-chart recorder (Bruck AC 200) and also on magnetic tape (Ampex PR 2200) for storage and subsequent detailed analysis of the variables under study. In addition, the dye-dilution curves were recorded on an X-Y recorder (Hewlett-Packard 40).

Design of experiment. The experiment was conducted with the subject in the supine position on a bed, as provided with fixed shoulder supports to avoid isometric work in the arms at the subject's head. An electrically actuated cycle ergometer (Elema-Schöander) was attached to the bed frame in such a way as to be at the level of the bed. The subject kept his feet on the pedals also in the resting condition. In the isometric exercise, subjects were thoroughly familiarized with the subjective sensations of leg exercise combined with PPB at the pressure levels to be used subsequently in the experimental study. In one of these sessions the individual aerobic power was determined using the nomogram of Astrand (1960). The experimental protocol (Fig. 1) comprised 3 parts. In the first, the responses to PPB during rest with the subject resting, with 4 min exposures to PPB at +15 and +30 cm H_2O . In the 2nd part of the protocol, separated by a 45 min recovery period, the subject exercised for 15 min at a low aerobic power with and without PPB at 15 and 30 cm H_2O . The sequence of the two parts was retained among the subjects. The exercise run without PPB served to give individual data on spontaneous base-line status of the variables under study occurring during steady-state exercise at the 4th through the 13th min. By compensating for these base-line shifts it was possible to study the changes produced by PPB during exercise. The data reported here refer to the last min in each condition (Fig. 1).

Calculations. Heart rate and mean arterial pressure were averaged during the inscription of each indicator on curve. Total peripheral resistance was calculated as the difference between mean arterial and mean venous pressure (mmHg) divided by cardiac output ($\text{ml} \cdot \text{min}^{-1}$), left ventricular work (watt) as the product of cardiac output ($\text{l} \cdot \text{min}^{-1}$) and the difference between mean arterial and mean central venous pressure (mmHg), divided by 430 l. The statistical significance of differences between mean values were tested by applying the *t*-test for the intrasubject differences (cf. Fisher 1946).

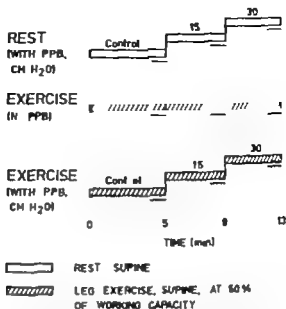


Fig. 1. Protocol showing the conditions of the experiment. PPB = continuous positive-pressure breathing. Middle portion of diagram signifies exercise at normal airway pressure. All data given in this table to periods indicated by the short horizontal lines appearing under each condition.

Results

The subjects were allowed to breathe spontaneously throughout the protocol with no instructions given regarding respiratory rate or depth of breath. Since all subjects had previously been familiarized with PPB at rest and during exercise, it can be assumed that factors such as apprehension or anxiety were not involved in the responses observed. The absence of emotional factors is borne out by the resting heart rates (Table II). Subjects only reported moderate respiratory discomfort at the end of PPB at 30 cm H₂O in the resting condition, whereas PPB during exercise was experienced as less unpleasant than at rest.

Respiratory and gas exchange variables Table I shows the changes observed in respiratory and blood gas variables, at rest and during exercise, as the airway pressure was raised to 15 and 30 cm H₂O. During PPB at 30 cm H₂O in the resting condition, the respiratory minute volume increased in all subjects, average increase amounting to 10.6 liters per minute (147%), and being almost entirely due to enlargement of the tidal volume. Fig. 2 shows that the PPB-induced hyperventilation and consequent arterial hypocapnia that occurred at rest were not present in the exercise condition. exercise-induced changes in respiratory minute volume, blood gases, and acid-base balance were not significantly affected by (cf. Table I).

Cardiovascular variables Table II gives the data obtained for cardiovascular variables with a change in the airway pressure from normal to 15 and 30 cm H₂O at rest and during exercise. PPB at 30 cm H₂O caused a curtailment of cardiac output averaging 22% at rest and during exercise, respectively. It is evident from Table II and Fig. 3 that these changes were due to reductions in stroke volume, which were not fully compensated by the concurrent increases in heart rate. The calculated central blood volume fell

I. Changes in respiratory variables, blood gases and acid-base balance produced by positive airway pressures (15 and 30 cm H₂O) in the resting condition and during leg exercise at 50 per cent of aerobic working capacity

No.	Rest			Exercise			Diff. Exercise— Rest at normal airway pressure
	Control	+15 cm H ₂ O ^a	+30 cm H ₂ O ^a	Control	+15 cm H ₂ O ^a	+30 cm H ₂ O ^a	
res. volume, l/TPS	7.58 ±0.49	3.78 ±2.00	10.64 ±2.29 ^a	44.09 ±4.40	1.29 ±1.06	1.28 ±1.45	36.51 ±3.91
volume, l	0.507 ±0.033	0.291 ±0.135	0.691 ±0.186	1.925 ±0.130	0.345 ±0.217	0.420 ±0.201	1.418 ±0.127 ^a
rate, mm ²	15.5 ±0.9	0.5 ±1.1	11.8 ±0.9	22.6 ±0.9	-2.2 ±1.7	-3.1 ±2.5	7.4 ±1.0 ^{a**}
Pa	91.4 ±1.9	17.6 ±4.9 ^a	24.3 ±6.4	93.2 ±2.8	2.1 ±2.5	0.4 ±4.1	1.8 ±1.7
PO ₂	39.8 ±1.5	-3.7 ±1.5 ^{a**}	-9.6 ±2.2 ^{a**}	40.3 ±1.6	-1.2 ±0.6	-0.2 ±1.0	0.5 ±0.6
EL	7.390 ±0.008	0.049 ±0.014 ^{a**}	0.089 ±0.024	7.353 ±0.010	0.010 ±0.005	-0.010 ±0.006	-0.037 ±0.004 ^{a**}
lactate l	24.5 ±0.3	0 ±0.3	0.1 ±0.4	22.6 ±0.5	-0.1 ±0.2	-0.6 ±0.4	-1.9 ±0.2 ^{a**}

are means ± S.E. (n = 8) ^a and ^{a**} denote p < 0.05, p < 0.01 and p < 0.001, respectively
since from control.

II. Changes in circulatory variables produced by positive airway pressures (15 and 30 cm H₂O) in the resting condition and during leg exercise at 50 per cent of aerobic working capacity

No.	Rest			Exercise			Diff. Exercise— Rest at normal airway pressure
	Control	15 cm H ₂ O ^a	+30 cm H ₂ O ^a	Control	+15 cm H ₂ O ^a	30 cm H ₂ O ^a	
res. rate, mm ²	63.2 ±2.4	4.6 ±2.4	16.1 ±4.6	122.9 ±2.8	5.0 ±1.8	0.3 ±2.4	59.7 ±3.6
ac output, ml	8.76 ±0.40	-1.23 ±0.46	1.93 ±0.79 ^a	18.12 ±0.82	-1.46 ±0.67	3.96 ±0.91 ^{a**}	9.36 ±1.00 ^{a**}
EL volume, l	140.5 ±6.1	27.5 ±5.5 ^{a**}	54.5 ±11.2 ^a	148.6 ±5.7	-16.1 ±6.8	-34.9 ±9.5	8.1 ±4.6
mean press., mm	93.5 ±2.9	3.6 ±1.4	7.8 ±2.1	122.9 ±4.5	11.8 ±2.0	5.3 ±2.1	24.4 ±3.5 ^{a**}
res. press., l ₂ O	5.8 ±0.3	6.3 ±0.5 ^{a**}	15.3 ±1.0 ^{a**}	5.7 ±0.6	10.5 ±0.9 ^a	21.5 ±1.3	-0.1 ±0.5
hr blood volume, l	1.150 ±0.09	180 ±67 ^a	-291 ±108	1.097 ±0.113	-91 ±71	-243 ±95 ^a	347 ±80 ^{a**}
per cent. oxy, %	1.82 ±0.16	0.41 ±0.10 ^a	0.09 ±0.13	4.93 ±0.31	-0.75 ±0.26	1.62 ±0.18 ^{a**}	3.11 ±0.37 ^a
per cent. reoxy, %	0.654 ±0.027	0.137 ±0.025 ^{a**}	0.375 ±0.021 ^{a**}	0.323 ±0.022	0.015 ±0.013	0.073 ±0.025	-0.269 ±0.034

are means ± S.E. (n = 8) ^a and ^{a**} denote p = 0.05, p = 0.01, and p = 0.001, respectively
since from control.

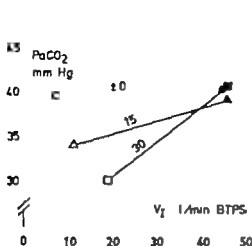


Fig. 2

Fig. 2. Ventilatory and P_aCO_2 responses to exercise at normal airway pressure and during pressure breathing at +15 and +30 cm H_2O . Open symbols refer to the resting, filled symbols to the exercise one. Mean values for 8 subjects.

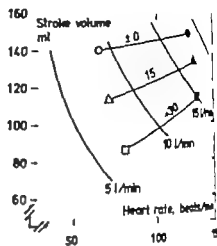


Fig. 3

Fig. 3. Changes in heart rate and stroke volume with transition from rest to exercise at normal airway pressure and during pressure breathing at +15 and +30 cm H_2O . Curved lines indicate different levels of cardiac output. Symbols as in Fig. 2.

of 20% with PPB at 30 cm H_2O in the resting condition, the corresponding reduction at exercise was significantly smaller, 13%. Left ventricular work decreased by 37% in response to PPB at 30 cm H_2O in the resting condition, and by 33% during exercise.

Mean arterial pressure was increased by an average of 8 mmHg ($p < 0.01$) with PPB at 30 cm H_2O in the resting condition; the corresponding increase during exercise was 5 mmHg ($p < 0.05$). At the same airway pressure, the mean central venous pressure was increased an average of 15 cm H_2O at rest and by 22 cm H_2O during exercise ($p < 0.001$ in both cases for the difference in response being < 0.01). Calculated total peripheral resistance increased by 42% with PPB at 30 cm H_2O in the resting condition and by 22% with the same airway pressure during exercise, the difference in response being statistically significant ($p < 0.01$).

Discussion

Continuous PPB in the resting condition, as employed in the present experiments, constitutes a well-defined form of functional stress that activates a multitude of partially interrelated reflexes in both the respiratory and cardiovascular systems. Basically respiration is shifted from an inspiratory act at normal airway pressure to an expiratory act during PPB and can be predicted from the pressure-volume diagram of the respiratory system (Rahn *et al.* 1960). The elevated intrathoracic pressure induced by PPB imposes an obstruction to inflow of blood from regions outside the trunk, with peripheral pooling and associated curtailment of cardiac output as the primary circulatory effects. We set out to explore some of the mechanisms involved in human respiratory and cardiovascular adaptation to dynamic exercise influenced by the simultaneous stress of PPB.

Respiratory responses. The occurrence of marked hyperventilation and respiratory

In response to continuous PPB at rest confirms observations in earlier work on the PPB to increase the alveolar oxygen tension during flight at high altitude (cf. Otis, n and Rahn 1955). The mechanism underlying the PPB-induced hyperventilation is own; it cannot be studied in anesthetized animals where even low levels of PPB reduction of ventilation or even temporary apnea (cf. Bjurstedt 1953). Considerable anal muscle activity was presumably involved in the expiratory efforts. Increased of this kind in animals has been shown to be a special reflex routed through the Bjurstedt 1953 Bishop 1964). The observed hyperventilation may have been the of such reflex drive beyond metabolic needs, but the involvement of other reflex nisms, or higher neural centers secondary to subjective respiratory discomfort, cannot eaded.

rees the ventilatory drive during PPB at rest showed great variability at rest, the tage change in ventilation at 30 cm H₂O ranging from -22 to +29% it was strikingly and by exercise. Thus, the corresponding range was -21 to +14% during exercise, oup mean ventilation in this condition being neither increased nor depressed. Also e alues for arterial blood gases and pH were essentially the same under PPB at 15) cm H₂O as they were with normal airway pressure. Hence, judging from the respira- esponses alone, subjects' exercise tolerance appeared to be little affected, if at all, B at the levels employed. Furthermore, it is likely that the average lung volume, which resting condition is enlarged by PPB (cf. Agostoni 1962), was reduced by the greater tory efforts during exercise and thus shifted towards normal. This may be inferred the fact that for a given increase in airway pressure, central venous pressure rose to a level during exercise than it did at rest, the difference averaging 6 cm H₂O ($p < 0.01$) PPB at 30 cm H₂O (cf. Table II and Fig. 4). Assuming that the central venous pressure ally follows the intrathoracic (cf. Otis, Rahn and Feen 1946), an elevation of the t pressure would occur if less of the applied airway pressure was absorbed by the as would be the case at a smaller lung volume. However, part of the 6 cm H₂O dif- ce in central venous pressure elevation may have been due to an increase of the right ranslational pressure in the exercise condition secondary to the action of the leg muscle

Effect of PPB on cardiac adaptation to exercise There is indication that in conscious at rest the average abdominal-thoracic pressure difference is positive during continuous up to 30 cm H₂O at which level this pressure difference becomes zero (Agostoni 1962). does not lead to venous pooling in the abdomen as long as the intrathoracic pressure is than the abdominal. Thus, there is reason to believe that in the present experiments PPB at rest, venous pooling was essentially restricted to regions outside the cavities of neck. Pooling in abdominal veins would be even less likely in PPB during exercise, in this condition the intermittent, downward depressions of the diaphragm associated the hyperpnea of exercise compresses the veins of the abdomen more forcefully than at, thus giving boost to the circulation by helping to move blood toward the heart. PPB at 15 and 30 cm H₂O caused cancellations of cardiac output by 18 and 22%, respec- y in the resting condition. This agrees well with the results of other investigators (cf. and Kleck 1963). The corresponding reductions during exercise were 8 and 21%.

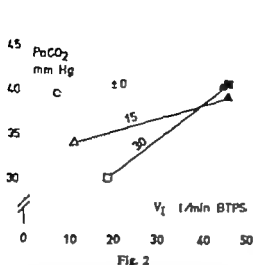


Fig. 2

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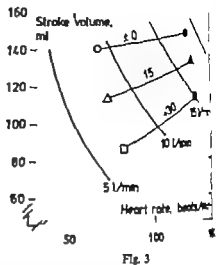


Fig. 3

Fig. 3. Changes in heart rate and stroke volume with transition from rest to exercise at normal pressure and during pressure breathing at +15 and +30 cm H_2O . Curved lines indicate different levels of cardiac output. Symbols as in Fig. 2.

of 20% with PPB at 30 cm H_2O in the resting condition the corresponding reduction during exercise was significantly smaller, 13%. Left ventricular work decreased by 37% in response to PPB at 30 cm H_2O in the resting condition and by 33% during exercise.

Mean arterial pressure was increased by an average of 8 mmHg ($p < 0.01$) with PPB at 30 cm H_2O in the resting condition the corresponding increase during exercise was 5 mmHg ($p < 0.05$). At the same airway pressure, the mean central venous pressure was increased an average of 15 cm H_2O at rest, and by 22 cm H_2O during exercise ($p < 0.001$ in both cases for the difference in response being < 0.01). Calculated total peripheral resistance increased by 42% with PPB at 30 cm H_2O in the resting condition, and by 22% with the same airway pressure during exercise, the difference in response being statistically significant ($p < 0.01$).

Discussion

Continuous PPB in the resting condition as employed in the present experiments, constitutes a well-defined form of functional stress that activates a multitude of partially interrelated reflexes in both the respiratory and cardiovascular systems. Basically respiration is initiated from an inspiratory act at normal airway pressure to an expiratory act during PPB and can be predicted from the pressure-volume diagram of the respiratory system (Rahn *et al.* 1961). The elevated intrathoracic pressure induced by PPB imposes an obstruction to inflow of blood from regions outside the trunk, with peripheral pooling and associated curtailment of cardiac output as the primary circulatory effects. We set out to explore some of the mechanisms involved in human respiratory and cardiovascular adaptation to dynamic exercise influenced by the simultaneous stress of PPB.

Respiratory responses The occurrence of marked hyperventilation and respiratory

in response to continuous PPB at rest confirms observations in earlier work on the PPB to increase the alveolar oxygen tension during flight at high altitude (cf Otis, and Rahn 1955). The mechanism underlying the PPB-induced hyperventilation is *own* cannot be studied in anesthetized animals where even low levels of PPB reduction of ventilation or even temporary apnea (cf Bjurstedt 1953). Considerable linal muscle activity was presumably involved in the expiratory efforts; increased of this kind in animals has been shown to be a special reflex routed through the Bjurstedt 1953 Bishop 1964). The observed hyperventilation may have been the of such reflex drive beyond metabolic needs, but the involvement of other reflex *rems*, or higher neural centers secondary to subjective respiratory discomfort, cannot *aded*

rest the ventilatory drive during PPB at rest showed great variability. At rest, the *age* change in ventilation at 30 cm H₂O ranging from -22 to +295, it was strikingly *in* by exercise. Then, the corresponding range was -21 to +14% during exercise, *deep* mass ventilation in this condition being neither increased nor depressed. Also *e* values for arterial blood gases and pH were essentially the same under PPB at 15 *l* cm H₂O as they were with normal airway pressure. Hence, judging from the respira-*responses* alone, subjects' exercise tolerance appeared to be little affected, if at all *B* at the levels employed. Furthermore, it is likely that the average lung volume, which *resting* condition is enlarged by PPB (cf Agostoni 1962), was reduced by the greater *tory* efforts during exercise and thus shifted towards normal. This may be inferred *he* fact that for a given increase in airway pressure, central venous pressure rose to a *level* during exercise than it did at rest, the difference averaging 6 cm H₂O ($p < 0.01$) *PPB* at 30 cm H₂O (cf Table II and Fig. 4). Assuming that the central venous pressure *ally* follows the intrathoracic (cf Otis, Rahn and Fenn 1946), an elevation of the *r* pressure would occur if less of the applied airway pressure was absorbed by the *is* could be the case at smaller lung volume. However part of the 6 cm H₂O dif-*erence* in central venous pressure elevation may have been due to an increase of the right *transmural* pressures in the exercise condition secondary to the action of the leg muscle

effects of PPB on cardiac adaptation to exercise There is indication that in conscious *at* rest the average abdominal-thoracic pressure difference is positive during continuous *up to* 30 cm H₂O at which level this pressure difference becomes zero (Agostoni 1962). *does* not lead to venous pooling in the abdomen as long as the intrathoracic pressure is *than* the abdominal. Thus, there is reason to believe that in the present experiments *PPB* at rest *enous* pooling was essentially restricted to regions outside the cavities of *rank*. Pooling in abdominal veins would be even less likely in PPB during exercise, *in* this condition the intermittent, downward depressions of the diaphragm associated *the* hyperpnea of exercise compress the veins of the abdomen more forcefully than *at*, thus giving boost to the circulation by helping to move blood toward the heart. *PPB* at 15 and 30 cm H₂O caused curtailments of cardiac output by 14 and 22%, respec-*tively* in the resting condition. This agrees well with the results of other investigators (cf *arn* and Sackel 1960). The corresponding reductions during exercise were 8 and 21%.

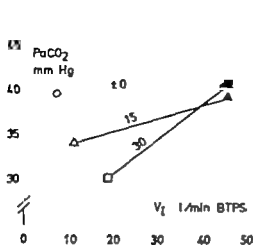


Fig. 2

Fig. 2. Ventilatory and PaCO_2 responses to exercise at normal airway pressure and during pressure breathing at +15 and +30 cm H_2O . Open symbols refer to the resting, filled symbols to the exercise and mean values for 8 subjects.

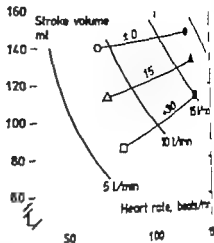


Fig. 3

Fig. 3. Changes in heart rate and stroke volume with transition from rest to exercise at normal airway pressure and during pressure breathing at +15 and +30 cm H_2O . Curved lines indicate decreases in cardiac output. Symbols as in Fig. 2.

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During the ventilatory drive during PPB at rest showed great variability. At rest, the average change in ventilation at 30 cm H₂O ranging from +22 to +295 l/min. It was strikingly reduced by exercise. Thus, the corresponding range was -21 to +14% during exercise, deep mean ventilation in this condition being neither increased nor depressed. Also the slopes for arterial blood gases and pH were essentially the same under PPB at 15 cm H₂O as they were with normal airway pressure. Hence, judging from the respiratory responses alone, subjects' exercise tolerance appeared to be little affected, if at all, by the levels employed. Furthermore, it is likely that the average lung volume, which in the resting condition is enlarged by PPB (cf Agostoni 1962), was reduced by the greater ventilatory efforts during exercise and thus shifted towards normal. This may be inferred from the fact that for a given increase in airway pressure, central venous pressure rose to a higher level during exercise than it did at rest, the difference averaging 6 cm H₂O ($p < 0.01$) at PPB at 30 cm H₂O (cf Table II and Fig. 4). Assuming that the central venous pressure normally follows the intrathoracic (cf Orla, Rahn and Fenn 1946), an elevation of the central venous pressure would occur if less of the applied airway pressure was absorbed by the lungs than would be the case at a smaller lung volume. However, part of the 6 cm H₂O difference in central venous pressure elevation may have been due to an increase of the right transpulmonary pressure in the exercise condition secondary to the action of the leg muscle

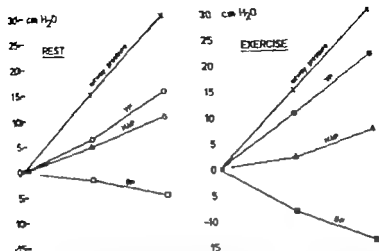
Effect of PPB on cardiac adaptation to exercise. There is indication that in conscious subjects at rest the average abdominal-thoracic pressure difference is positive during continuous PPB up to 30 cm H₂O at which level this pressure difference becomes zero (Agostoni 1962). This does not lead to venous pooling in the abdomen as long as the intrathoracic pressure is higher than the abdominal. Thus, there is reason to believe that in the present experiments PPB at rest-induced venous pooling was essentially restricted to regions outside the cavities of the trunk. Pooling in abdominal veins would be even less likely in PPB during exercise, in this condition the intermittent, downward depressions of the diaphragm associated with the hyperpnea of exercise compress the veins of the abdomen more forcefully than at rest, thus giving boost to the circulation by helping to move blood toward the heart. At 15 and 30 cm H₂O caused curtailments of cardiac output by 14 and 22%, respectively, in the resting condition. This agrees well with the results of other investigators (cf Wahren and Sandler 1960). The corresponding reductions during exercise were 8 and 21%.

The mode of adaptation of cardiac dynamics to exercise is altered by PPB. Fig. 3 is the well known fact that at normal airway pressure moderate leg exercise in the supine position increases cardiac output almost entirely by an increase in heart rate. When the same exercise during PPB, the initial stroke volume was smaller than it was at normal airway pressure. However, with leg exercise it increased, and the demands on the heart were met by a greater contribution of the stroke volume the higher the airway pressure. This response pattern can best be explained by assuming that during exercise the blood volume initially pooled in the legs by PPB is redistributed into the central venous system by the action of the leg muscle pump. Such redistribution of blood volume is similar to what has been shown to occur in exercise when the circulation is challenged by increased stress in the upright body position (Bevegård, Holmgren and Jonsson 1960) or by an increased gravitational load (Rosenhamer 1967). With regard to the behavior of the stroke volume, it can thus be concluded that leg exercise improves the circulatory response to the imposed stress in much the same way whether such stress is caused by PPB or by the action of the G factor.

In the calculation of the central blood volume (CBV), values for mean transit time were employed that referred to the transport of dye from the right atrium to the sampling site in the pinna of the ear. The data for calculated CBV given in Table II therefore are, in principle, from the same uncertainty as is always the case when a peripheral sampling site is employed. As shown by Gleason *et al.* (1959) and Marshall and Shepherd (1961) calculated CBV will be overestimated when flow in arteries leading to the sampling site is relatively slower than that through other systemic circuits. Since in exercise the flow of blood supplying the head is greatly diminished in relation to that distributed to the working muscles, the observed increase in CBV in response to exercise at normal airway pressure, which averaged 347 ml, may therefore in part or even wholly have been due to an increase in the so-called systemic component.

When the airway pressure is increased in the resting condition, a displacement of blood volume out of the thorax into distensible peripheral veins can be expected and has been estimated by Fenn *et al.* (1947) on the basis of a caudad shift of weight in humans lying on a tilt table. There are no data available on PPB-induced changes in CBV from experiments where the dye-dilution technique has been employed with the sampling site relatively near the aortic valves. In the present experiments, PPB at 30 cm H₂O reduced calculated CBV by an average of 291 ml at rest; the corresponding reduction during exercise being 101 ml (p in both cases < 0.05). No measurements are available as to the effects of PPB on the relative distribution of blood flow to the head and other systemic vascular beds. For this reason the calculated reductions in CBV due to PPB should be interpreted with caution. However, since the sampling site was relatively near the root of the aorta, and it is assumed that PPB did not induce any large shifts in the relative distribution of blood flow in systemic circuits, the observed reductions in calculated CBV would seem to be representative for the true changes that occurred in the blood volume contained in the heart and lungs.

Responses of vascular pressures to PPB. In the present experiments the driving pressure in the abdominal and cerebrospinal cavities the driving pressure



Changes in measured mean arterial (MAP = \square) and central venous pressures (CVP = \blacksquare) and in calculated driving pressure in the systemic circuits of the body DP (= MAP - CVP) as produced by increases in airway pressure. MAP represents transmural arterial pressure at the carotid baroreceptors, DP representing pressure at the aortic baroreceptors.

between measured mean arterial and central venous pressures (MAP and CVP respectively) is the circulation outside these cavities venous blood from the periphery encounters elevated pressure during PPB leading to increased transmural venous pressures with consequent peripheral pooling of blood. Also in this part of the circulation DP is the difference between MAP and CVP assuming that the pressures in the veins where they enter the trunk approximate CVP. Hence, Fig. 4 represents the average changes observed in MAP and CVP and in those of DP in all systemic circuits as the airway pressure was increased from normal to 15 and 30 cm H₂O. It can be seen that DP was reduced by PPB, the respective values at 30 cm H₂O averaging 4 mmHg at rest, and 11 mmHg during exercise ($p < 0.01$). During PPB the heart operates from a higher than normal pressure base. This means that the left ventricle can cut down its work, if it is to deliver the normal pressure to regions outside the trunk. In fact, PPB at 30 cm H₂O reduced left ventricular work by 37 and 33% at rest and during exercise, respectively even though the mean arterial pressure (relative to the atmospheric) was increased in both conditions (cf Table II). In Fig. 4 the difference between mean arterial pressure (MAP) and the driving pressure (DP) signifies the reduction in the share in MAP contributed by the contractions of the heart.

In the resting condition PPB at 30 cm H₂O resulted in an increase in MAP (relative to atmospheric), averaging 8 mmHg ($p < 0.01$), which is in accordance with earlier observations in conscious man (Dorn and Fenn 1947). This pressure rise represents the increase in transmural pressure that occurred at the carotid baroreceptors. There was a concurrent calculated total peripheral resistance amounting to no less than 4.4%, on the average, below of the vasoconstriction known to be an important compensating mechanism during PPB (Fenn et al. 1947 Blair Glover and Kidd 1959). This effect cannot be explained as a reduction in mean pressure at the carotid baroreceptors, which would tend to reduce

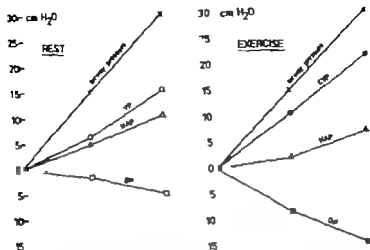
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Responses of vascular pressures to PPB In the systemic circulation in the abdominal and cerebrospinal cavities the driving pressure (DP) during PPB

(thorax)



Changes in measured mean arterial (MAP = 8) and central venous pressures (CVP = 6) and in driving pressure in the systemic circuits of the body DP (\sim MAP - CVP) as produced by increasing airway pressure. MAP represents transmural arterial pressure at the carotid baroreceptors, DP depending pressure at the aortic baroreceptors.

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peripheral resistance. Fig. 4 shows, on the other hand, that the aortic baroreceptors have been involved. For DP represents not only the driving pressure, it also approximates transmural aortic pressure assuming that CVP follows the intrathoracic pressure (cf. *et al* 1946). Thus, a fall of DP induced by PPB would lead to a reduced impulse traffic to the aortic baroreceptors and a consequent increase in peripheral resistance. PPB also reduces central blood volume (cf. Table II) and presumably also the effective filling pressure of the heart due to the displacement of blood volume into peripheral veins. The vasoconstrictor effect known to result in man from unloading of intrathoracic low-pressure baroreceptors (Zoller *et al* 1972, Johnson *et al* 1974) can therefore be assumed to have acted in concert with a similar effect from aortic reflex influence. Also, the pulse pressure was presumably diminished at both the carotid and aortic baroreceptors secondary to the decreased cardiac output, which would have added to this effect (cf. Ead, Green and Neil 1961, recent review see Kirchheim 1976). The fact that DP fell somewhat means that the observed marked increase in total peripheral resistance was not enough to compensate for the peripheral circulatory effect of PPB, that of decreased cardiac output. However, with the support of the increased intrathoracic pressure transmitted along the arteries leading out of the thorax, the vasoconstrictor defense was effective in actually increasing arterial pressure relative to the atmospheric, as illustrated by the response of MAP in Fig. 4. These responses were observed in the remarkable situation of opposite transmural pressure changes in the right ventricle and the aortic arch (cf. MAP and DP in Fig. 4), a salient feature in PPB.

It is of interest to note, in this connection, the behavior of the arterial pressure in anesthetized animals during PPB. In such animals, even a small increase in airway pressure by 10–15 cm H₂O leads to a fall in arterial pressure instead of the increase commonly observed in conscious man. This discrepancy has been resolved since the important effect of the abdominal pressure in the arterial pressure response has been recognized (Björk 1953, Bjurstedt, Wood and Åström 1953). The reduced muscle tone in the abdomen, due to general anesthesia, or brought about by voluntary relaxation in conscious man, favors venous pooling in the abdomen in addition to that in the periphery and so leads to a failure of arterial pressure homeostasis.

The PPB-induced rise in total peripheral resistance at 30 cm H₂O decreased from an average of 42% at rest to 22% during exercise (p for the difference <0.01), the corresponding reductions in cardiac output being similar in the two conditions (22 and 21%, respectively). Exercise may therefore be regarded as having caused a weakening of the reflex vasoconstrictor influence arising from PPB-induced unloading of aortic and intrathoracic low-pressure baroreceptors. With regard to the arterial baroreceptor reflexes, certain investigators have concluded that these reflexes are reset or inhibited during exercise (for recent review, see McRitchie *et al* 1976). On the other hand, a reduction of PPB-induced vasoconstriction occurring in exercise may only indicate that the vasoconstrictor outflow to resting vessels is normally resulting from dynamic exercise (cf. Blair, Glover and Roddie 1961, Bengtsson and Shepherd 1966) is but little influenced by the additional stimulus of PPB.

Cerebral circulation. As first shown by Hamilton, Woodbury and Harper (1944), intrathoracic pressure as induced in a variety of respiratory maneuvers is freely transmitted to the cerebrospinal fluid due to the movements of loose tissue and fluid through the

ral foramina and into the cerebrospinal canal (for review see Scharpey-Schafer 1965).
ore, PPB-induced elevation of central venous pressure will not lead to damming up
od in intracranial veins. It is conceivable that PPB at a sufficiently high level could
the driving pressure in cerebral circuits to such an extent that respiratory and cardio-
ar effects would result from underperfusion of medullary centers. In the present
however the reductions in cerebral driving pressure, represented by the fall of DP
A were too small to make such effects likely
conclusion, sustained PPB in the supine body position reduced the driving pressure in
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References

- 1954, E. Diaphragm activity and thoracoabdominal mechanics during positive pressure breathing.
J. appl. Physiol. 1962, 17 215-220
- 1950, I. Aerobic work capacity in man and oxygen utilization with special reference to age. *Acta physiol. scand.*
1950, 2, Suppl. 149
- 1954, T. and E. WIGERTZ, A digital computer for automatic breath-by-breath calculations of respiratory
changes. *Rep. Lab. Arter. Neural Med., Karol. Inst. Stockholm* March 1966 14 pp.
- 1954, B. S. and J. T. SCHARPEY, Reaction in man of resistance and capacity vessels in forearm and
and in leg exercise. *J. appl. Physiol.* 1966, 21 123-132
- 1954, S. A. HOLMES and B. JENSEN, The effect of body position on the circulation at rest and
during exercise, with special reference to the influence on the stroke volume. *Acta physiol. scand.* 1960,
279-294
- 1954, B. Reflex control of abdominal muscles during positive-pressure breathing. *J. appl. Physiol.* 1964,
224-232
- 1954, H. Influence of the abdominal muscle tone on the circulatory response to positive pressure
breathing in unanesthetized dogs. *Acta physiol. scand.* 1953, 29 145-162.
- 1954, H. G. ROBINSON and E. TROPEA, Lower body negative pressure and effects of autonomic
nerve blockade on cardiovascular responses. *Acta physiol. scand.* 1977 99 353-360.
- 1954, H. E. H. WOOD and A. ASTROM, Cardiovascular effects of raised airway pressure. *Acta physiol.*
scand. 1953, 29 190-201
- 1954, R. D. A. W. E. GLOVER and B. S. L. KATO, The effect of continuous positive and negative pressure
breathing upon the resistance and capacity blood vessels of the human forearm and hand. *Clin. Sci.*
1959 18 9-16
- 1954, R. D. A. W. E. GLOVER and I. C. ROBINSON, Vasomotor responses in the human arm during leg exercise.
Scandinavian Rev. 1961 9 264-274
- 1954, R. J. and W. O. FRY, The effect of varying pulmonary pressures on the arterial pressures in man
and unanesthetized dogs. *J. clin. Invest.* 1957 26 460-467
- 1954, H. W. J. H. GILFILL and E. NEIL, A comparison of the effects of pulsatile and non-pulsatile blood
flow through the carotid sinus on the reflexogenic activity of the sinus baroreceptors in the cat. *J. Physiol.*
London 1952, 112 509-519.
- 1954, R. W. E. A. B. OTTE, H. RAHN, L. E. CHADWICK and A. H. HIGGINS, Displacement of blood from
the lungs by pressure breathing. *Amer. J. Physiol.* 1947 151 258-269
- 1954, R. A. *Statistical Methods for Research Workers*, 10th Ed. Edinburgh, Oliver and Boyd Ltd. 1948,
154 pp.
- 1954, L. W. L. J. M. BACON, D. E. MILLER and H. D. McIVER, A major pitfall in the interpretation of
the central blood volume. *Clin. Res.* 1959 7 227

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In vitro effects of growth hormone on protein synthesis and amino acid transport in the rat diaphragm after acute hypophysectomy

By

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Abstract

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Effects of growth hormone (GH) *in vitro* on phenylalanine-¹⁴C incorporation to assess protein synthesis and on endogenous 1-¹⁴H accumulation to measure amino acid transport in the diaphragm muscle were investigated 2, 6 and 24 h after hypophysectomy or sham-operation. In hypophysectomized rats protein synthesis was depressed. GH *in vitro* was without effect 2 h after hypophysectomy but stimulated protein synthesis 6 and 24 h after the operation. Six hours after hypophysectomy amino acid transport was enhanced and further stimulated by GH. After 24 h amino acid transport was depressed and stimulated to normal levels by GH. Six hours after sham-operation protein synthesis was depressed, but was stimulated by GH. After 24 h protein synthesis was normalized and GH was without effect. GH did not influence amino acid transport after sham-operation. Plasma levels of GH were undetectable after hypophysectomy, markedly depressed 2 and 6 h after sham-operation, but normal after 24 h. We concluded that tissue responsiveness to GH develops a few hours after hypophysectomy.

Studies on the effects of growth hormone (GH) on protein metabolism have been performed in animals hypophysectomized for several days or weeks (Kostyo and Nutting 1974). Administration of GH to hypophysectomized rats, results in a prolonged (> 24 h) stimulation of protein synthesis in skeletal muscle (Kostyo and Nutting 1973). The amino acid transport is, however, only transiently stimulated by GH and is then "refractory" to additional GH (or 24-48 h) (Hjalmarsson and Ahren 1967 b). In contrast to the findings in hypophysectomized rats, the effects of exogenous GH on protein metabolism in tissues of normal rats are poor or inconsistent (Kostyo and Nutting 1974). Since tissues in the normal animal are exposed to endogenous GH, it is possible that the poor effects of exogenous GH in normal animals are due to the long-lasting effects of circulating GH. Hypophysectomy is, however, accompanied by a hormonal imbalance which might cause structural and metabolic aberrations in the tissues (Eckert 1976). Florini and Brewer (1966) demonstrated that the capacity of thigh muscle ribosomes of chronically hypo-

- HAMILTON, W. F., R. A. WOODBURY and H. T. HARPER JR., Arterial, cerebrospinal and venous pressure in man during cough and strain. *Amer J Physiol* 1944, 141, 42-50.
- JOHNSON, J. M., L. B. ROWELL, M. NIEDERBERGER and M. EITMAN, Human splanchnic and forearm constrictor responses to reductions of right atrial and aortic pressures. *Circulat Res* 1974, 34, 58.
- KILBURN, K. H. and H. O. SIEKER, Hemodynamic effects of continuous positive airway pressure breathing in normal man. *Circulat Res* 1960, 8, 660-669.
- KIRCHGEISS, H. R., Systemic arterial baroreceptor reflexes. *Physiol. Rev* 1976, 56, 100-177.
- LEFANT, C. and B. J. HOWELL, Cardiovascular adjustments in dogs during continuous pressure breathing. *J appl. Physiol* 1960, 15, 425-428.
- LINDBORG, B., TH. ÖDMAN and O. WIGERTZ, A beat-to-beat heart-rate meter with digital and linear analog output. *Rep Lab Appl Med, Karol Inst Stockholm* 1969.
- LUTT, U. C., Aviation physiology—the effects of altitude. In *Handbook of Physiology Responses to Altitude* D. C. Am. Physiol. Soc. 1965 Sect. 3, II 1099-1145.
- MCCRITCHIE, R. J., S. F. VATNER, D. BOETTCHER, G. R. HEYNDRIKX, T. A. PATRICK and E. BARNETT, Role of arterial baroreceptors in mediating cardiovascular response to exercise. *Amer J Physiol* 1970, 230, 85-89.
- MARSHALL, R. J. and J. T. SHEPHERD, Interpretation of changes in central blood volume and volume during exercise in man. *J cl Invest* 1961, 40, 375-385.
- OTIS, A. B., H. RAHN and W. O. FENN, Venous pressure changes associated with positive pressure breathing: the relationship to the distensibility of the lung. *Amer J Physiol* 1946, 146, 367-377.
- OTIS, A. B., M. B. SHELDON JR. and H. RAHN, The effects of positive pressure breathing in man upon pneumotachogram, ventilation and alveolar gas pressure. *WADC Techn. Rep* 53-57, 1. *Patterson AFB Ohio* 1955, 4-32.
- RAHN, H., A. B. OTIS, L. E. CHADWICK and W. O. FENN, The pressure-volume diagram of the thorax in man. *Amer J Physiol* 1946, 146, 161-178.
- ROSENHAMER, O., Influence of increased gravitational stress on the adaptation of cardiovascular and respiratory function to exercise. *Acta physiol scand* 1967, Suppl. 267.
- SHARPEY-SCHAEFER, E. P., Effect of respiratory acts on the circulation. In *Handbook of Physiology* G. H. H. Washington, D.C. Am. Physiol. Soc. 1965 Sect. 2, III, 1875-1886.
- ZOLLER, R. P., A. L. MARRAS, F. M. ARABOUD, W. G. SCHMID and D. D. HEISTAD, The role of arterial baroreceptors in reflex vasoconstrictor responses in man. *J clin. Invest* 1972, 51, 2967-2972.

In vitro effects of growth hormone on protein synthesis and amino acid transport in the rat diaphragm after acute hypophysectomy

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Abstract

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Effects of growth hormone (GH) *in vitro* on phenylalanine-¹⁴C incorporation to assess protein synthesis and on [³H] leucine incorporation to measure amino acid transport in the diaphragm muscle were investigated 2, 6 and 24 h after hypophysectomy or sham-operation. In hypophysectomized rats protein synthesis was depressed. GH *in vitro* was without effect 2 h after hypophysectomy but stimulated protein synthesis 6 and 24 h after the operation. Six hours after hypophysectomy amino acid transport was enhanced and further stimulated by GH. After 24 h amino acid transport was depressed and stimulated to normal levels by GH. Six hours after sham-operation protein synthesis was depressed, but was stimulated by GH. After 24 h protein synthesis was normalized and GH was without effect. GH did not influence amino acid transport after sham-operation. Plasma levels of GH were undetectable after hypophysectomy, markedly depressed 2 and 6 h after sham-operation, but normal after 24 h. It is concluded that tissue responsiveness to GH develops few hours after hypophysectomy.

Studies on the effects of growth hormone (GH) on protein metabolism have been performed in animals hypophysectomized for several days or weeks (Kostyo and Nutting 1974). Administration of GH to hypophysectomized rats, results in a prolonged (>24 h) stimulation of protein synthesis in skeletal muscle (Kostyo and Nutting 1973). The amino acid transport into muscle is, however, only transiently stimulated by GH and is then "refractory" to additional GH for 24-48 h (Hjalmarsson and Ahren 1967b). In contrast to the findings in hypophysectomized rats, the effects of exogenous GH on protein metabolism in tissues of normal rats are poor or inconsistent (Kostyo and Nutting 1974). Since tissues in the normal rat are exposed to endogenous GH, it is possible that the poor effects of exogenous GH in tissues from normal animals are due to the long lasting effects of circulating GH. Hypophysectomy is, however, accompanied by a hormonal imbalance which might cause structural and metabolic aberrations in the tissues (Everitt 1976). Florini and Bruer (1966) demonstrated that the capacity of thigh muscle ribosomes of chronically hypo-

hypophysectomized rats to catalyze protein synthesis was markedly reduced. Further, a single dose of GH was found to restore the capacity of the ribosomes to that of normal rats after a time lag of several hours. The yield of ribosomal RNA paralleled these findings. These findings suggest that the long duration of the effects of GH on protein metabolism in hypophysectomized rats might not be representative for normal rats but merely a reflection of a functional impairment in the protein synthetic machinery due to the chronic lack of GH.

In the present study the effects of exogenous GH on protein metabolism of the skeletal muscle of the rat were studied at short time intervals after hypophysectomy. It is known that lack of pituitary hormones would cause structural changes in the peripheral tissues.

Materials and Methods

Animals. Female rats, 30 days old (80–95 g) of the Sprague-Dawley strain purchased from Altus, Stockholm, Sweden, were used in all experiments. The animals were housed in a room in which temperature (25°C) and humidity (50–60%) were kept constant. The rats were fed standard commercial diet (Type R3, Anticimex, Stockholm), and tap water *ad libitum*. The rats were hypophysectomized under ether anesthesia by the standard parapharyngeal approach (1930) that has been used for many years in this laboratory (e.g. Hjalmarsson 1968). A corresponding sham-operation—that is all steps in the surgical procedure except removing the pituitary—were performed. After the operation the rats were kept in the animal room and received food *ad libitum* and in addition 50% glucose solution *ad libitum*. One group of the rats were untreated and used as controls. 6–8 h after the operation the rats were killed for *in vitro* experiments.

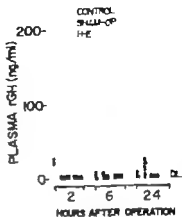
Radioactive materials. Radioactive substances, obtained from New England Nuclear Corp. (Boston, Mass.), were used at the following activities and molarities: α -amino-isobutyric acid- ^3H (4.8 $\mu\text{Ci}/\text{mM}$), 0.2 mM L-phenylalanine- ^3H (4.0), 0.03 $\mu\text{Ci}/\text{mM}$, 0.03 mM.

Hormone. Bovine growth hormone [NIH bovine GH 18 (bGH)] with a potency of 0.8 IU/mg was supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Incubation procedure. Rats were rapidly decapitated and blood was collected in chilled test tubes. The livers were removed and the kidneys were weighed. The muscles were removed and placed in beakers for subsequent glucose and GH determination. The rectal hemidiaphragms were prepared as described by Knobl (1959) and washed for 10 min at 37°C in 25 ml Krebs bicarbonate buffer, pH 7.4 (Oxoid), equilibrated with 95% O_2 and 5% CO_2 . After the washing period, 10 individual hemidiaphragms were transferred to other flasks containing 10 ml of fresh KRB with 10 natural amino acids at normal plasma levels (e.g. Isaksson *et al.* 1971) and the labelled amino acids. The medium for one number of each pair of muscles also contained bGH (5 $\mu\text{g}/\text{ml}$). The hemidiaphragms were incubated at 37°C for 60 min and then the muscles were weighed and homogenized in 1.5 ml 5% perchloric acid. The amount of protein was determined according to Lowry *et al.* (1951). Radioactivity measurements were performed as previously described (Albertsson-Wikland and Isaksson 1976). The extent of amino acid transport was calculated as the ratio of the distribution ratio (cpm/ml intracellular water/cpm/ml incubation medium) and the values for control were previously determined (Hjalmarsson and Ahren 1967a, Albertsson-Wikland and Isaksson 1976). Alanine incorporation was expressed as dpm of ^3H incorporated/100 μg of muscle protein.

Plasma GH determination. Blood collected from the trunk of decapitated rats was immediately centrifuged and plasma was separated, stored and frozen at -30°C until assay. Plasma GH was measured by immunoradiometric assay using a double antibody technique as previously described (Edén *et al.* 1977). As a preparation, highly purified rat GH, especially prepared by Dr A. E. Wilhelm with an activity of 100 IU/mg, was used. The same hormone was labelled with ^{125}I according to the lactoperoxidase method (Th. Johansson 1971). Monkey anti-rGH serum (A rGH 5-3) was generously supplied by the National Institute of Arthritis, Metabolism and Digestive Diseases. Precipitating antibodies against normal monkey serum were raised in rabbits according to the method of Vitale *et al.* (1971). Triplicates of plasma (1:3 dilutions (5, 10 and 20 $\mu\text{l}/\text{tube}$)) were used for the determination of the rGH concentration. The volume of plasma was kept constant in all samples. Equivalent amounts of plasma from hypophysectomized rats. The detection limit of the assay was 0.05 ng rGH/tube and the standard curve was linear between 5–200 ng/ml plasma (50 $\mu\text{l}/\text{tube}$).

levels of GH in normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats. Rats were rapidly bled and blood collected from the trunk. Plasma GH assayed by radioimmunoassay. D.L. represents the detection limit (2 S.D. above zero dose on the standard curve). Each point is the plasma level of GH in individual rats. There were no measurable GH levels in hypophysectomized rats. In sham-operated rats GH levels were undetectable 2 h after surgery but significantly lower than in normal rats 6 h after surgery (Wilcoxon test) but not significantly different from levels 24 h after surgery.



Statistical procedures. Values are given as means \pm S.E. Comparisons were made by paired *t*-test, or when 2 values are compared, by analysis of variance followed by Student Newman-Keuls test between individual groups (Woolf 1966). *P*-values less than 0.05 were considered significant and by Wilcoxon plasma GH levels were compared Wilcoxon non-parametric test as used (Colquhoun

Results

Levels of GH were measured in all rats. There were no detectable levels of GH 2, 6 h after hypophysectomy. Sham-operation caused a marked reduction of plasma GH (Fig. 1). There were no detectable levels of GH 2 h after sham-operation. 6 h post-operatively 4 out of 12 animals had measurable plasma GH levels but levels were significantly lower compared to normal controls. 24 h after sham-operation GH levels were almost equal to normal levels.

Blood glucose was also measured in all rats (Table I). The table shows that there was a decrease in blood glucose 2 h postoperatively in sham-operated and hypophysectomized rats. 6 h postoperatively there were no significant differences between the three groups. After hypophysectomy blood glucose levels were slightly decreased.

Effects of bGH (5 μ g/ml) *in vitro* on the incorporation of phenylalanine 3 H into protein and on the accumulation of AIB- 3 H in diaphragms of normal, sham-operated and hypophysectomized rats 6 h postoperatively are shown in Fig. 2. GH did not stimulate these

Table I. Blood glucose in normal, sham-operated and hypophysectomized rats 2, 6 and 24 h postoperatively.

Hours after operation	Blood glucose (mM)		
	normal rats	sham-operated rats	hypophysectomized rats
2			
6			
24			
2	5.53 \pm 0.43	4.74 \pm 0.24	6.50 \pm 0.46*
6	5.43 \pm 0.39	6.00 \pm 0.21	5.22 \pm 0.32
24	5.85 \pm 0.48	5.79 \pm 0.26	4.03 \pm 0.41

* *P* < 0.05 compared with analysis of variance.

physysectomized rats to catalyze protein synthesis was markedly reduced. A single dose of GH was found to restore the capacity of the ribosomes to that of sham after a time lag of several hours. The yield of ribosomal RNA paralleled these findings suggesting that the long duration of the effects of GH on protein metabolism in hypophysectomized rats might not be representative for normal rats but merely a reflection of a functional impairment in the protein synthetic machinery due to the chronic lack of pituitary hormones.

In the present study the effects of exogenous GH on protein metabolism of the skeletal muscle of the rat were studied at short time intervals after hypophysectomy. The lack of pituitary hormones would cause structural changes in the peripheral tissues

Materials and Methods

Animals. Female rats, 30 days old (80–95 g) of the Sprague-Dawley strain purchased from Axon, Stockholm, Sweden, were used in all experiments. The animals were housed in a room in which temperature was maintained from 05.00 to 19.00 h and temperature (25°C) and humidity (50–60%) were held constant. The rats were fed a standard commercial diet (Type R3, A. C. L. Malm, Stockholm), and tap water *ad libitum*. The rats were hypophysectomized under ether anesthesia by the standard parapharyngeal approach (Johansson 1930) that has been used for many years in this laboratory (e.g. Hjalmarsson 1968). A corresponding group of rats were sham-operated—that is, all steps in the surgical procedure except removing the pituitary were performed. After the operation the rats were kept in the animal room and received food *ad libitum* and 1 addition 50% glucose solution *ad libitum*. One group of the rats were untreated and were killed 6 or 24 h after the operation; the rats were killed for *in vivo* experiments.

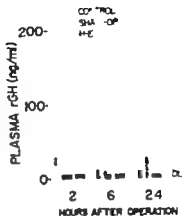
Radioactive material. Radioactive substances, obtained from New England Nuclear Corp., Boston, Mass., were used at the following activities and molarities: α -amino-isobutyric acid-1-¹⁴C (AB 100) 0.2 μ Ci/ml 0.2 mM; L-phe-tylalanine-3-¹⁴C (U) 0.05 μ Ci/ml 0.08 mM.

Hormone. Bovine growth hormone (NIH-bovine GH 18 (bGH)) with a potency of 0.8 IU/mg as determined by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Incubation procedure. Rats were rapidly decapitated and blood was collected in chilled test tubes for subsequent glucose and GH determination. Intact hemidiaphragms were prepared (Kobayashi and Knobil 1959) and washed for 10 min at 37°C in 25 ml Krebs bicarbonate buffer pH 7.4 (Krebs) liberated with 95% O₂ and 5% CO₂. After the washing period, individual hemidiaphragms were transferred to other flasks containing 10 ml of fresh KRBB with all natural amino acids at normal plasma levels (Johansson 1971) and the labelled amino acids. The medium for one chamber of each pair of muscles was bGH (5 μ g/ml). The hemidiaphragms were incubated at 37°C for 60 min and then the muscles were blotted in buffer, weighed and homogenized in 1.5 ml 5% perchloric acid. The amount of protein was determined according to Lowry *et al.* (1951). Radioactivity measurements were performed as previously described (Albertsson-Wikland and Isaksson 1976). The extent of amino acid transport was calculated as the distribution ratio (cpm/ml intracellular water/cpm/ml incubation medium) using values for cellular water content previously determined (Hjalmarsson and Ahlström 1967; Albertsson-Wikland and Isaksson 1976). Alanine incorporation was expressed as dpm of ¹⁴C incorporated/100 μ g of muscle protein.

Plasma GH determination. Blood collected from the trunk of decapitated rats was immediately centrifuged and plasma was separated, stored and frozen at -30°C until assay. Plasma GH was measured by a double antibody technique as previously described (Edén *et al.* 1978). Antiserum preparation, highly purified rat GH especially prepared by Dr A. E. Wilhelm with a specific activity of 10⁶ IU/mg was used. The same hormone was labelled with ¹²⁵I according to the lactoperoxidase method (Th. Johansson 1971). Monkey anti-rGH-serum (A-rGH S-3) was generously supplied by the M. Precipitating antibodies against normal monkey serum were raised in rabbits according to the procedure of Vajkalis *et al.* (1971). Triplicates of plasma in 3 dilutions (5, 10 and 20 μ l/tube) were used for determination of the rGH concentration. The volume of plasma was kept constant in all samples to equivalent amounts of plasma from hypophysectomized rats. The detection limit of the assay was 0.05 ng GH/tube and the standard curve was linear between 5–200 ng/ml plasma (10 μ l/tube).

Plasma levels of GH in normal, sham-operated (SHAM) and hypophysectomized (H-E) rats. Rats were rapidly bled and blood collected from the trunk. Plasma GH assayed by radioimmunoassay. D.L. represents the detection limit above zero done as the standard curve. Each point is the plasma level of GH in individual rats. There is no measurable GH-levels in hypophysectomized rats. Hypophysectomized GH-levels were undetectable 2 h after surgery, significantly lower than in normal rats 6 h after surgery (Wilcoxon test) but not significantly different from levels 24 h after surgery.



statistical procedures. Values are given as mean \pm S.E. Comparisons are made by paired *t*-test, or more than 2 values were compared, by analysis of variance followed by Student-Newman-Keuls *q*-test between individual groups (Thomlin 1966). *P*-values less than 0.05 were considered significant in this study. When plasma GH levels were compared Wilcoxon's non-parametric test was used (Colquhoun

Results

Plasma levels of GH were measured in all rats. There were no detectable levels of GH 2 h after hypophysectomy. Sham-operation caused a marked reduction of plasma GH (Fig. 1). There were no detectable levels of GH 2 h after sham-operation. 6 h post-operatively 4 out of 12 animals had measurable plasma GH levels but levels were significantly lower compared to normal controls. 24 h after sham-operation GH levels were almost equal to normal levels.

Blood glucose was also measured in all rats (Table I). The table shows that there was a significant increase in blood glucose 2 h postoperatively in sham-operated and hypophysectomized rats. 6 h postoperatively there were no significant differences between the three groups. 24 h after hypophysectomy blood glucose levels were slightly decreased.

The effects of bGH (5 μ g/ml) *in vitro* on the incorporation of phenylalanine- 3 H into protein and on the accumulation of AIB- 3 H in diaphragms of normal, sham-operated and hypophysectomized rats 2 h postoperatively are shown in Fig. 2. GH did not stimulate these

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physysectomized rats to catalyze protein synthesis was markedly reduced. Further single dose of GH was found to restore the capacity of the ribosomes to that of control after a time lag of several hours. The yield of ribosomal RNA paralleled these findings. These findings suggest that the long duration of the effects of GH on protein metabolism in hypophysectomized rats might not be representative for normal rats but merely a reflection of a functional impairment in the protein synthetic machinery due to the chronic lack of pituitary hormones.

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Radioactive materials. Radioactive substrates, obtained from New England Nuclear Corp. (Boston, Mass.), were used at the following activities and molarities: α -amino-³H-isobutyric acid-1-³H (AIB) 10 μ Ci/ml, 0.2 mM; L-phenylalanine-¹⁴C (U), 0.05 μ Ci/ml, 0.08 mM.

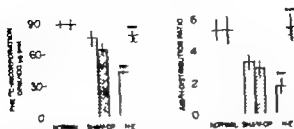
Hormone. Bovine growth hormone [NIH-bovine GH 18 (bGH)] with a potency of 0.8 IU/mg was purchased from the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD).

Incubation procedure. Rats were rapidly decapitated and blood was collected in chilled test tubes. The blood was allowed to clot and the serum was separated and stored at -20°C until assay. Plasma GH was measured by a double antibody technique as previously described (Edén *et al.* 1978). A radioimmunoassay preparation, highly purified rat GH, especially prepared by Dr A. E. Wilhelm with an activity of 2.1 μ g/mg, was used. The same hormone was labelled with ¹²⁵I according to the lactoperoxidase method (Theodorsson 1971). Monkey anti-rGH serum (A-rGH 3-3) was generously supplied by the NIA. Precipitating antibodies against normal monkey serum were raised in rabbits according to the procedure of Vaitaitis *et al.* (1971). Triplicates of plasma in 3 dilutions (5, 10 and 20 μ l/tube) were used for determination of the rGH concentration. The volume of plasma was kept constant in all samples by equivalent amounts of plasma from hypophysectomized rats. The detection limit of the assay was 0.1 ng plasma (0.05 ng rGH/tube) and the standard curve was linear between 5–200 ng/ml plasma (0.1 ng rGH/tube).

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24 H POST-OP

□ CONTROL □ GH 5 µg/100 ml



Effect of GH *in vivo* on phenylalanine-¹⁴C incorporation (left) and AIB-³H accumulation (right) in rats of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 24 h after surgery. Animals were incubated for 60 min with and without bGH (5 µg/100 ml). The incorporation of phenyl-¹⁴C into protein and the distribution ratio of AIB-³H were determined as described in text. Each bar represents the mean of 10-12 observations. Vertical lines represent S.E.

0.01 or normal control with analysis of variance

0.001 or normal control with analysis of variance

0.001 or corresponding control with paired t-test

in diaphragms of both sham-operated and hypophysectomized rats, but had no effect in the control group of normal rats. In the hypophysectomized group, GH *in vivo* restored phenylalanine ¹⁴C incorporation to normal levels while the effect was less pronounced but significant in the sham-operated group. The distribution of AIB-³H was significantly altered in the sham-operated group while the distribution was markedly increased in diaphragms of hypophysectomized rats. GH *in vivo* did not significantly affect amino acid transport in normal or sham-operated rats whereas the distribution of AIB-³H was further elevated in diaphragms of hypophysectomized rats.

Postoperatively (Fig. 4) the incorporation of phenylalanine ¹⁴C and the accumulation of AIB-³H was markedly decreased in diaphragms of hypophysectomized rats. Exposure to GH *in vivo* resulted in a marked stimulation of both of these parameters. Thus, the incorporation rate of phenylalanine ¹⁴C and accumulation rate of AIB-³H were close to that in diaphragms of normal rats. In the sham-operated rats the distribution ratio of AIB-³H was slightly decreased but the incorporation of phenylalanine-¹⁴C was unchanged compared to diaphragms of normal rats. GH did not significantly affect any of these two parameters in the sham-operated group.

Discussion

Immediate metabolic changes following hypophysectomy have received little attention. Keet and Russell (1968) found an increase in nitrogen excretion and a decrease in the liver glycogen content 24 h after hypophysectomy in the rat. They further found that GH therapy restored these parameters to control values suggesting that the observed phenomenon was partially due to lack of GH.

The present study clearly shows that the processes regulating the rate of amino acid transport and protein synthesis become responsive to exogenous GH a short time after hypophysectomy. In the present experiments significant effects of GH on these two para-

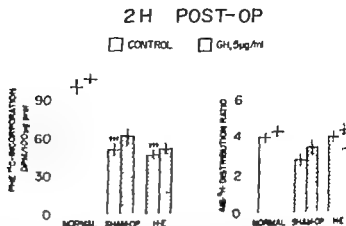


Fig. 2. Effect of GH *in vivo* on phenylalanine- ^{14}C incorporation (left) and AIB- ^3H accumulation in diaphragms of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 2 h after surgery. Hemidiaphragms were incubated for 60 min with and without bGH (5 µg/ml). The incorporation of phenylalanine- ^{14}C in muscle protein and the distribution ratio of AIB- ^3H were determined as described in Methods. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

† $p < 0.05$ vs. normal control with analysis of variance
 ††† $p < 0.001$ vs. normal control with analysis of variance

two parameters in any of the groups studied. However, there was a marked decrease in control levels of phenylalanine- ^{14}C incorporation in diaphragms of sham-operated and hypophysectomized rats compared to normal rats. The accumulation of AIB- ^3H was also decreased in the control diaphragms of sham-operated rats but not in diaphragms of hypophysectomized rats.

6 h after surgery the incorporation of phenylalanine- ^{14}C was still reduced in both groups (Fig. 3). However, GH *in vitro* stimulated the incorporation of the amino acid

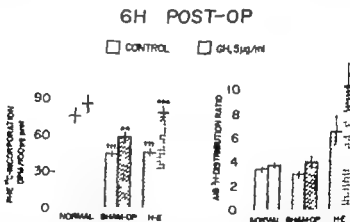


Fig. 3. Effect of GH *in vitro* on phenylalanine- ^{14}C incorporation (left) and AIB- ^3H accumulation in diaphragms of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 6 h after surgery. Hemidiaphragms were incubated for 60 min with and without bGH (5 µg/ml). The incorporation of phenylalanine- ^{14}C in muscle protein and the distribution ratio of AIB- ^3H were determined as described in Methods. Each bar represents the mean of 10–12 observations. Vertical lines represent S.E.

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 ††† $p < 0.001$ vs. normal control with analysis of variance
 † $p < 0.05$ vs. corresponding control with paired t-test
 †† $p < 0.01$ vs. corresponding control with paired t-test
 ††† $p < 0.001$ vs. corresponding control with paired t-test

24 H POST-OP

□ CONTROL □ GH $1 \mu\text{g}/100 \text{ ml}$



Effect of GH *in vivo* on phenylalanine-¹⁴C incorporation (left) and AIB-³H accumulation (right) in rats of normal, sham-operated (SHAM-OP) and hypophysectomized (H-E) rats 24 h after surgery. Diaphragms are incubated for 60 min with and without h-GH (5 $\mu\text{g}/100 \text{ ml}$). The incorporation of phenylalanine-¹⁴C into muscle protein and the distribution ratio of AIB-³H are determined as described in Table 1. Each bar represents the mean of 10-12 observations. Vertical lines represent \pm S.E.

* 0.01 *vs* normal control with analysis of variance

** 0.001 *vs* normal control with analysis of variance

*** 0.001 *vs* corresponding control with paired *t*-test

in diaphragms of both sham-operated and hypophysectomized rats, but had no effect in the control group of normal rats. In the hypophysectomized group, GH *in vivo* restored phenylalanine-¹⁴C incorporation to normal levels while the effect was less marked but significant in the sham-operated group. The distribution of AIB-³H was significantly altered in the sham-operated group, while the distribution was markedly decreased in diaphragms of hypophysectomized rats. GH *in vivo* did not significantly alter amino acid transport in normal or sham-operated rats whereas the distribution of AIB-³H was further elevated in diaphragms of hypophysectomized rats.

Postoperatively (Fig. 4) the incorporation of phenylalanine-¹⁴C and the accumulation of AIB-³H was markedly decreased in diaphragms of hypophysectomized rats. Exposure to GH *in vivo* resulted in a marked stimulation of both of these parameters. Thus, the incorporation rate of phenylalanine-¹⁴C and accumulation rate of AIB-³H were close to that in diaphragms of normal rats. In the sham-operated rats the distribution ratio of AIB-³H was slightly depressed but the incorporation of phenylalanine-¹⁴C was unchanged compared with diaphragms of normal rats. GH did not significantly affect any of these two parameters in the sham-operated group.

Discussion

The immediate metabolic changes following hypophysectomy have received little attention. Kee and Russell (1968) found an increase in nitrogen excretion and a decrease in the liver glycogen content 24 h after hypophysectomy in the rat. They further found that GH therapy restored these parameters to control values suggesting that the observed phenomenon was partially due to lack of GH.

The present study clearly shows that the processes regulating the rate of amino acid transport and protein synthesis become responsive to exogenous GH a short time after hypophysectomy. In the present experiments significant effects of GH on these two para-

meters were seen at 6 and 24 h after hypophysectomy. These results suggest that the effects of endogenous GH on protein synthesis and amino acid transport in the rat are of short duration in contrast to the long lasting effects of GH on these parameters previously reported in tissues of hypophysectomized rats (Kostyo and Nutting 1971; Larsson and Ahren 1967b). This hypothesis is further supported by recent results made in our laboratory using the diaphragm muscle of normal 18-day-old rats (Albertsson-Wikland and Isaksson 1976, 1978). In these experiments the duration of the metabolic effects of GH on protein synthesis and amino acid transport was considerably longer than that previously reported in hypophysectomized rats.

From the present experiments it is not clear to what extent the changes in protein metabolism observed could be ascribed to lack of GH. The surgical trauma per se may have caused changes in tissue metabolism, which in turn might change the tissue response to GH. The possibility that a substantial amount of GH leaked out into the blood during the operation, and then influenced peripheral metabolism for a long time after the operation, was ruled out, since plasma levels of GH are low during hypophysectomy and remain undetectable after the operation (unpublished observation).

GH levels in plasma were low or undetectable also in sham-operated rats for at least 6 h after the surgical trauma, which is in accordance with previous observations which have shown that unspecific stress inhibits the release of GH (Takahashi *et al.* 1971; Doss 1973/74; Terry *et al.* 1976). In spite of low or undetectable levels of GH in both sham-operated and hypophysectomized animals for at least 6 h after the operation, the distribution ratio of AIB was markedly elevated in the hypophysectomized group while there was no change in the distribution ratio of AIB in the sham-operated group. Interestingly, similar observations have been made in the small intestine of the rat by Levitan *et al.* (1977) who reported an initial increase in amino acid transport after hypophysectomy followed by a marked decrease. The cause of the altered amino acid uptake after hypophysectomy compared to that after sham operation is not clear, but this phenomenon indicates that factors besides low GH levels in plasma are involved in the metabolic changes reported after hypophysectomy.

Recent studies by Martin and co-workers (Tannenbaum and Martin 1976) have shown that GH is secreted in a pulsatile pattern in the rat. The short duration of the stimulatory effect of GH on protein synthesis in young normal animals opens the possibility that brief peaks of GH intermittently stimulate protein synthesis. Such a temporal relationship between peaks in plasma concentration of GH and protein synthesis in skeletal muscle has not been proposed (Isaksson *et al.* 1978). Further studies are, however, required to substantiate this hypothesis.

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References

- ON-WIGLAND, K. and O. ISAKSSON, Development of responsiveness of young normal rats to hormone. *Metabolism* 1976, 25, 747-759.
- ON-WIGLAND, K. and O. ISAKSSON, Time-course of the effect of growth hormone on streptozotocin and monosaccharide transport and on protein synthesis in diaphragm of young normal rats. *Endocrinology* 1978, 102, 1445-1451.
- SONN, D., *Lectures on Biochemistry*, Clarendon Press, Oxford 1971.
- WILSON, W. J., SCHWARTZ, M. D., HUTCHINS, L. E., SCHWARTZ, C. T. and C. T. WILSON, Daily variation in rat growth hormone concentration and the effect of stress on periodicity. *Neuroendocrinology* 1973/74, 13.
- WILSON, W. J., ALBERTSON-WIGLAND and O. ISAKSSON, Plasma levels of growth hormone in female rats of various ages. *Acta endoc.* (Kbh.) 1976, 88, 676-690.
- WILSON, W. J., Hypophysectomy and aging in the rat. In *Hypothalamus primary and aging*. Eds. Everitt, J. and J. A. Burger, Charles C. Thomas Publisher, Springfield, 1976, 64-85.
- WILSON, W. J. and C. B. BURTON, Amino acid incorporation into protein by cell-free system from rat skeletal muscle. V. Effects of pituitary growth hormone on activity of ribosomes and on amino acid polymerization. *Biochemistry* 1966, 5, 1870-1876.
- WILSON, W. J., *Effects of growth hormone on the metabolism of the isolated rat diaphragm*. Oxonius tryck AB, Göteborg, Thessa, 1966.
- WILSON, W. J. and K. ARNTZ, Sensitivity of the rat diaphragm to growth hormone. I. In vitro and in vivo effects of growth hormone on amino acid transport. *Acta endoc.* (Kbh.) 1967, 54, 645-662.
- WILSON, W. J. and K. ARNTZ, Sensitivity of the rat diaphragm to growth hormone. II. Early and late effects of growth hormone on amino acid and protein uptake. *Acta endoc.* (Kbh.) 1967, 56, 347-358.
- WILSON, W. J., D. F. NUTTIN, J. L. KORTVO and C. R. REAGAN, Hourly variations in the plasma concentration of growth hormone and insulin and in amino acid uptake and incorporation into protein in diaphragm muscle of the rat. *Endocrinology* 1978, 102, 1430-1438.
- WILSON, W. J. and M. KUO, The effect of growth hormone on the in vitro incorporation of leucine-3-¹⁴C into the protein of rat diaphragm. *Endocrinology* 1979, 105, 395-401.
- WILSON, W. J. and D. F. NUTTIN, Acute in vivo effects of growth hormone on protein synthesis in various tissues of hypophysectomized rats and their relationship to the levels of thymidine factor and insulin in plasma. *Horm. Metab. Res.* 1973, 5, 167-172.
- WILSON, W. J. and D. F. NUTTIN, Growth hormone and protein metabolism. In *Handbook of Physiology*, IV, Part 2, Eds. Knobil, E. and W. H. Sawyer, American Physiol. Soc., Washington D.C., 1974, 219.
- WILSON, W. J., E. HAVTE, L. POORE and R. D. COLEMAN, Amino acid absorption and incorporation after hypophysectomy. *Amer. J. Physiol.* 1974, 126, 525-532.
- WILSON, W. J., O. H. M. J. ROBINSON, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin and phenol reagent. *J. Biol. Chem.* 1951, 193, 265-275.
- WILSON, W. J. and J. A. RUTELL, Effect of acute hypophysectomy and growth hormone on PFA mobilization, nitrogen excretion and cardiac glycogen in fasting rats. *Endocrinology* 1968, 83, 1162-1165.
- WILSON, W. J., D. C. N. EARL, A. BROADBENT, E. B. WOLPERT, K. E. GORE and L. R. JEFFERSON, Regulation of protein synthesis in heart muscle. I. Effect of amino acid levels on protein synthesis. *J. Biol. Chem.* 1966, 241, 2152-2162.
- WILSON, W. J., Hypophysectomy and replacement therapy in the rat. *Amer. J. Anat.* 1930, 45, 205-273.
- WILSON, W. J., W. H. DALGARADA and D. M. KIRBY, Regulation of immunoreactive growth hormone secretion in male rats. *Endocrinology* 1971, 88, 909-917.
- WILSON, W. J., G. E. and J. B. MARTIN, Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* 1976, 98, 562-570.
- WILSON, W. J., J. O. WILLOUGHBY, P. BEAZLEY and J. B. MARTIN, Antiserum to somatomedin prevents streptozotocin-induced inhibition of growth hormone secretion in the rat. *Science* 1976, 192, 565-567.
- WILSON, W. J. and R. G. JORDANSON, Enzymatic isolation of polypeptides with high specific activity. *Protein. Biophys. Acta (Amst.)* 1971, 251, 363-369.
- WILSON, W. J., J. B. ROBERTS, E. NICHOLAS and G. T. ROSE, A method for producing specific extracts of the endocrine system. *J. Clin. Endoc.* 1971, 33, 928-931.
- WILSON, W. J., *Principles of Biochemistry* (Ed. 1) D. van Nostrand Company Inc. Princeton, N.J. U.S.A., 1970.

Metabolic effects of blood flow restriction in adipose tissue

By

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Abstract

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The metabolic effect of blood flow restriction were studied in isolated blood-perfused canine omentum adipose tissue. Blood flow restriction (on the average to 20 per cent of control flow) was caused by mechanical clamping of the arterial inflow or by i.a. injections of methoxamine or angiotensin. Glucose uptake in the adipose tissue was reduced during blood flow restriction. This was partially compensated for by a period of increased glucose uptake following restoration of flow. Blood flow restriction also increased the venous lactate/pyruvate ratio. The basal lipolytic rate was decreased during blood flow restriction. Lipolysis induced by brief (3 min) sympathetic nerve stimulation (4 Hz) was not inhibited by blood flow restriction. The total amount of glycerol released from the tissue was unaffected. The rate was reduced during blood flow restriction but glycerol trapped within the tissue was apparently reutilized by the fat cells as it was released upon flow restoration. FFA outflow following nerve stimulation was, however, inhibited suggesting increased reutilization of FFA within the tissue. This increased utilization may ultimately be caused by the observed change in red/or.-balance and/or by the limited capacity (albumin) available during blood flow restriction. Three main conclusions may be drawn from the present results. Firstly plasma levels of glycerol and FFA do not necessarily reflect adipose tissue levels at given moment. Secondly the decreased adipose tissue blood flow seems to be a major cause of lowered FFA-levels during hemorrhage. Thirdly in contrast to hemorrhage even severe reduction of adipose tissue blood flow is insufficient to cause irreversible ischemic damage.

Key words. Adipose tissue, Blood flow, Esterification, Fatty acids, lipolysis.

Plasma levels of glycerol and FFA (non-esterified fatty acids) are commonly used indicators of lipolysis and are thought to closely reflect events in the adipose tissue. It has been reported that plasma levels of glycerol are unchanged and those of FFA actually decreased during hemorrhage in dogs (Rosell *et al* 1973) and rats (Farnebo *et al* 1977). This decrease in plasma FFA during hemorrhage, which is accompanied by intense sympatho-adrenal stimulation has been attributed to the severe reduction in adipose tissue blood flow (Rosell *et al* 1973). Kovách *et al* (1976) reduced blood flow to the unstimulated adipose tissue by 85% during 60 minutes. They found that the basal outflow of glycerol and FFA was reduced during flow reduction but that no irreversible damage had been caused by the experimental procedure. The ability of the adipose tissue to respond to a stimulus during conditions of blood flow restriction is never

d. We have therefore examined the influence of blood flow restriction, caused either by constrictor agents or by mechanical clamping of the arterial inflow on the response to standardized (Fredholm 1970) stimulation of the sympathetic nerves supplying the subcutaneous adipose tissue of the dog.

Methods

Experiments were performed on female mongrel dogs weighing 10-20 kg. The dogs were anesthetized with ketalar (30 mg/kg) with supplements as necessary and mechanically intubated. Subcutaneous tissue in the inguinal region was completely isolated from the surrounding tissue (Rosell 1966), for one artery, one vein and nerve containing sympathetic fibres. The vessels were cannulated with tubing and the adipose tissue was connected to the femoral circulation. Adipose tissue blood flow measured with dilution filled drop recorders on the arterial side and registered together with carotid blood pressure on a Grass 7B polygraph. Restriction of adipose tissue blood flow for 60-90 min was achieved by a) mechanical clamping of the arterial supply b) repetitive i.v. injections of 4-6 µg noradrenaline c) repetitive injections of 100-300 µg angiotensin (Hypertensin CIBA) although final doses of 3-10 µg were needed to maintain low flow. Nerve stimulation was performed by placing the sectioned nerve on bipolar silver electrodes giving electrical impulses at 4 Hz for 5 min with supra-maximal intensity (13 V) and duration (2 ms). Venous blood samples from the adipose tissue vein and arterial blood samples were collected into ice-cooled centrifuge tubes. Lactate and pyruvate concentrations determined in aliquots of whole blood with commercially available reagents (TCC, TCB, Boehringer Mannheim). After centrifugation aliquots of plasma were removed for the determination of glycerol (Laurell 1964), FFA (Laurell and Tabbutt 1967) and glucose (Glor, Kabi). Uptake or release rates of lactate, pyruvate, glycerol, FFA and glucose were calculated by multiplying the V-A-concentration difference with adipose tissue blood or plasma flow. The net release of glycerol or FFA was obtained by adding the basal release from the total amount released during and following sympathetic activation.

Results

Adipose tissue blood flow was reduced (on the average by 80 per cent of the flow during control conditions) during 90 min either by mechanical clamping of the arterial supply or

1 Net release of glycerol and FFA was calculated as the total amount released during and after sympathetic nerve stimulation. In the case of flow reduction the amounts mobilized after flow restoration have been excluded. 1 absolute terms (nmol/100 g tissue) the net release of glycerol was 11.6 ± 3.7 (control) and 14.6 ± 3.0 (flow reduction) and FFA as 33.6 ± 10.2 (control) and 7.5 ± 4.4 (flow reduction).

restriction	of control	Net release of control	
		Glycerol	FFA
control	26	104	49
control	37	108	24
noradrenaline	16	41	0
noradrenaline	8	112	0
noradrenaline	17	99	5
Ang	23	74	32
Ang	7	106	0
Ang	14	36	3
Ang	10	105	0
	18 ± 3	87 ± 10	12 ± 6

CLAMP

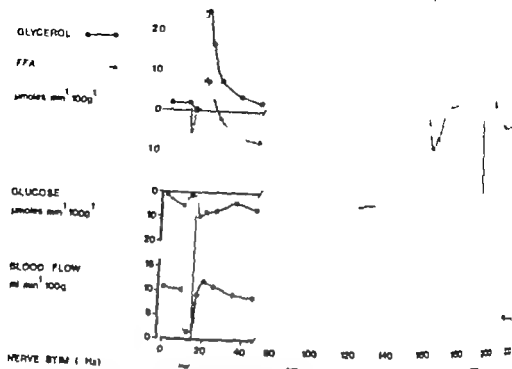


Fig. 1 Time course of changes in release/uptake rates of glycerol, FFA and glucose and blood flow during reduction of blood by mechanical clamping. Three consecutive sympathetic nerve stimulations were performed, the second one during reduction of blood by mechanical clamping.

by i.a. injections of methoxamine or angiotensin (Table I). Angiotensin was used in 2 expts. since a sustained blood flow reduction was difficult to obtain due to tachyphylaxis.

In each experiment three consecutive sympathetic nerve stimulations at 4 Hz for 5 s were performed, the first and the third serving as controls while the second stimulation was performed during the period of flow reduction (Fig. 1). There were no differences between the two control periods concerning uptake/release of glucose, lactate, glycerol or FFA. Furthermore, there were no differences in net glycerol or FFA release between the two control periods. Therefore, the events during flow reduction have been compared to the means of the two controls in each experiment.

The basal outflow of glycerol from the adipose tissue decreased somewhat during flow restriction (Fig. 1, Table II), even though there was a compensatory increase in V A glycerol concentration difference at lower blood flow rates (Fig. 2). The peak effect of glycerol following sympathetic nerve stimulation was delayed (Fig. 1) and was reduced by flow restriction (Fig. 1, Table II). The maximal V A concentration difference (following stimulation) for glycerol did not increase during flow restriction, indicating that the maximal transport capacity for glycerol is reached already after a 4 Hz nerve stimulation during control conditions. The net release of glycerol from the adipose tissue due to brief sympathetic nerve stimulations during blood flow restriction was, however, unchanged (Table I).

During control conditions there was a net uptake of FFA

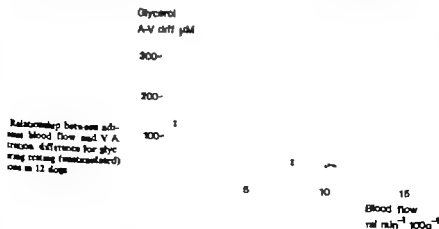
not stimulated

Mean release rates ($N=9$) of glycerol, FFA and glucose (A) at rest before sympathetic nerve stimulation (B) peak after sympathetic nerve stimulation and (C), the peak rate upon restoration of blood flow. I the rate of glucose uptake during period B the mean uptake during the whole 30 to 40 min period after sympathetic nerve stimulation as calculated.

Release rate ($\mu\text{mol min}^{-1} 100 \text{ g}^{-1}$)			
	A. Mean before stim.	B. Peak after stim.	C. Peak following flow restoration
of glycerol	0.18 ± 0.02 0.09 ± 0.02	3.02 ± 0.54 0.51 ± 0.19	— 0.58 ± 0.12
of FFA	-0.29 ± 0.11 -0.01 ± 0.04	3.35 ± 0.97 0.41 ± 0.23	— -0.37 ± 0.37
Mean after stim.			
of glucose	-4.2 ± 0.5 -1.9 ± 0.2	-4.1 ± 0.4 -2.6 ± 0.4	— -8.1 ± 1.3

Fig. 1 Table II), which was reduced by flow restriction. Both the peak outflow rate (Table II) and the net release of FFA (Table II) after sympathetic nerve stimulation were diminished by flow restriction. There was no compensatory increase in FFA upon restoration of blood flow (Fig. 1 Table II), in contrast to what was found for . Glucose uptake was reduced by approximately 50 per cent during blood flow restriction (Table II). After restoration of flow there was a compensatory increase in glucose which lasted 5 to 10 min (Fig. 1 Table II).

Under control conditions there was a net uptake of lactate in the adipose tissue amounting to $0.35 \mu\text{mol min}^{-1} 100 \text{ g}^{-1}$. Blood flow restriction converted this uptake into a release rate of $0.57 \pm 0.16 \mu\text{mol min}^{-1} 100 \text{ g}^{-1}$. As a further indication of anaerobic metabolism the lactate/pyruvate concentration ratio in the venous effluent from the adipose tissue increased from 15 ± 4 to 27 ± 8 .



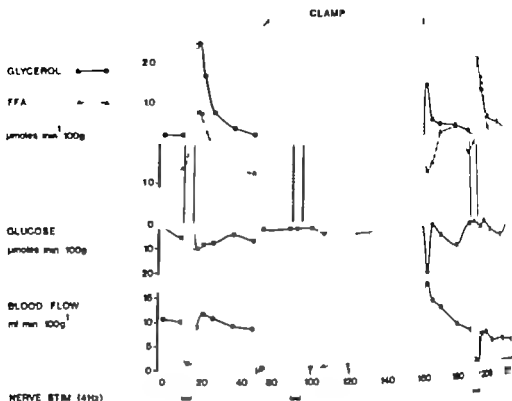


Fig. 1 Time course of changes in release/uptake rates of glycerol, FFA and glucose and blood flow in one experiment. Three consecutive sympathetic nerve stimulations were performed, the second one during reduction of blood by mechanical clamp ng.

by i.a. injections of methoxamine or angiotensin (Table I). Angiotensin was used in 2 expts. since a sustained blood flow reduction was difficult to obtain due to tachyphylaxis.

In each experiment three consecutive sympathetic nerve stimulations at 4 Hz for 30 s were performed, the first and the third serving as controls while the second stimulation was performed during the period of flow reduction (Fig. 1). There were no differences between the two control periods concerning uptake/release of glucose, lactate, glycerol or FFA. Furthermore, there were no differences in net glycerol or FFA release between the control periods. Therefore, the events during flow reduction have been compared to the means of the two controls in each experiment.

The basal outflow of glycerol from the adipose tissue decreased somewhat during blood flow restriction (Fig. 1 Table II) even though there was a compensatory increase in V A glycerol concentration difference at lower blood flow rates (Fig. 2). The peak outflow of glycerol following sympathetic nerve stimulation was delayed (Fig. 1) and markedly reduced by flow restriction (Fig. 1 Table II). The maximal V A concentration difference (following stimulation) for glycerol did not increase during flow restriction, indicating that the maximal transport capacity for glycerol is reached already after a 4 Hz nerve stimulation during control conditions. The net release of glycerol from the adipose tissue due to brief sympathetic nerve stimulations during blood flow restriction was, however, unchanged (Table I).

During control conditions there was a net uptake of FFA from the unstimulated adipose

Mean release rates ($N = 9$) of glycerol, FFA and glucose (A) at rest before sympathetic nerve stimulation (B) peak after sympathetic nerve stimulation and (C), the peak seen upon restoration of blood flow in the case of glucose uptake during period B the mean uptake during the whole 30 to 40 min period after sympathetic nerve stimulation was calculated.

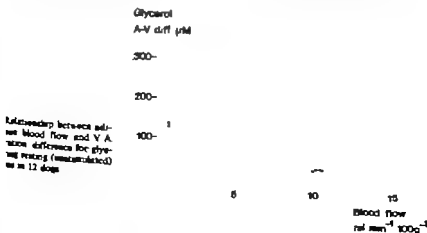
Release rate ($\mu\text{mol} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$)

A. Mean before stim. B. Peak after stim. C. Peak following flow restoration

l release	0.18 ± 0.02 0.09 ± 0.02	3.02 ± 0.54 0.51 ± 0.19	— 0.58 ± 0.12
d release	0.29 ± 0.11 -0.01 ± 0.04	3.33 ± 0.97 0.41 ± 0.28	— -0.37 ± 0.37
Mean after stim.			
d release	-4.2 ± 0.5 1.9 ± 0.2	-4.1 ± 0.4 -2.6 ± 0.4	— -8.1 ± 1.3

Fig. 1 Table II), which was reduced by flow restriction. Both the peak outflow rate (Table II) and the net release of FFA (Table II) after sympathetic nerve stimulation only diminished by flow restriction. There was no compensatory increase in FFA upon restoration of blood flow (Fig. 1 Table II), in contrast to what was found for . Glucose uptake was reduced by approximately 50 per cent during blood flow on (Table II). After restoration of flow there was a compensatory increase in glucose which lasted 5 to 10 min (Fig. 1 Table II).

ing control conditions there was a net uptake of lactate in the adipose tissue amounting $\pm 0.35 \mu\text{mol} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$. Blood flow restriction converted this uptake into a fraction rate of $0.57 \pm 0.16 \mu\text{mol} \cdot \text{min}^{-1} \cdot 100 \text{ g}^{-1}$. As a further indication of anaerobic metabolism the lactate/pyruvate concentration ratio in the venous effluent from the tissue increased from 15 ± 4 to 27 ± 8 .



Discussion

The present results confirm the opinion that adipose tissue blood flow is of import for the mobilization of lipolytic products, *i.e.* glycerol and FFA, from the tissue (cf. 1966 Fredholm 1970). A severe reduction of adipose tissue blood flow does not appear to impair the lipolytic response to sympathetic nerve stimulation, since the net release of glycerol was unchanged. On the other hand, the glycerol mobilization was delayed and a substantial part of the release occurred after the period of restricted flow. When blood flow is induced in a tissue with low blood flow glycerol may therefore be trapped in the tissue for very long periods of time before mobilization. This implies that observed plasma glycerol levels do not necessarily reflect the lipolytic rate in the adipose tissue at a given time. Himma-Hagen (1967) has enumerated several factors that tend to make conclusions concerning actions in adipose tissue from studies of plasma FFA and glycerol levels uncertain. The present findings seem to add yet another complicating factor to that list.

While blood flow restriction did not affect the total amount of glycerol released during a period of nerve stimulation (if sufficient time for sampling was allowed for) there was a reduction of the net release of FFA. This suggests that blood flow restriction has less effect on the re-utilization of fatty acids in the tissue.

The increased utilization of FFA during blood flow restriction is best explained by an increased rate of reesterification of newly formed fatty acids. Several factors may contribute to this. Firstly a low blood flow and thus a decrease in carrier capacity (*i.e.* albumin) will increase the concentration of FFA within the tissue. Secondly the increased lactate/pyruvate ratio indicates an elevated NADH/NAD⁺-ratio (Huckabee 1958). This will enhance the formation of α -glycerophosphate which is the other substrate required for triglyceride synthesis in the adipose tissue, as discussed previously (Fredholm 1971).

The present experiments show that the adipose tissue is not damaged by blood flow restriction during 60–90 min since stimulated glycerol and FFA-outflow as well as glucose uptake were almost identical during observation periods before and after blood flow restriction. Similarly Kovách *et al* (1976) found no irreversible damage to unstimulated adipose tissue when blood flow was reduced to 15% of control for 60 min by mechanical clamping. Thus restriction of adipose tissue blood flow to less than 20% of control does not produce evidence of ischemia.

These findings are in agreement with the findings that adipose tissue oxygen tension is only slightly reduced during mechanical clamping (Fredholm *et al* 1976). However, noradrenaline, causing both active vasoconstriction and metabolic activation, lowers adipose tissue oxygen tension substantially (Fredholm *et al* 1976). These differences may explain why experimental hemorrhage in contrast to a mere blood flow reduction may cause irreversible damage to adipose tissue (Kováč *et al* 1970, Fredholm and Fredholm 1974) since hemorrhage is accompanied by intense sympatho-adrenal activation.

With these considerations as a background it may seem puzzling that blood flow restriction reduces adipose tissue oxygen uptake (Fredholm *et al* 1976, Kovách *et al* 1976). We would like to propose that this decrease in oxygen uptake is caused by the simultaneous decrease in glucose uptake by the tissue, and hence by a decreased

in adipose tissue is to store, as economically as possible, surplus energy delivered to animals. It can be calculated that a major part of the substrate delivered to adipose *in situ* is used, not to subserve basic metabolic needs, but for synthesis, presumably triglycerides (Fredholm and Karlsson 1970, Fredholm *et al.* 1976). Thus, in this particular increased energy supply (by for example reducing blood flow) may primarily lead not to compromised cellular function but merely to a reduced rate of energy storage.

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References

- EDL, L.-O., B. B. FREDHOLM, B. HANSSON, P. HANSSON and L. WESTMAN, Cyclic AMP and metabolites in hemorrhagic shock of the rat. *Acta chir. scand.* 1977. 143. 9-14.
- EDL, B. B. Studies on the sympathetic regulation of circulation and metabolism in isolated canine subcutaneous adipose tissue. *Acta physiol. scand.* 1970. 80 Suppl. 354.
- EDL, B. B., The effect of lactate in canine subcutaneous adipose tissue *in situ*. *Acta physiol. scand.* 1971. 81. 110-122.
- EDL, B. B. and J. KARLSSON, Metabolic effects of prolonged sympathetic nerve stimulation in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1970. 80. 567-576.
- EDL, B. B. and A. FROBERG, High energy phosphate compounds in adipose tissue: The effect of orthostasis. *Acta physiol. scand.* 1974. 91. 165-171.
- EDL, B. B., B. LINCK, R. L. PARWITT and P. C. JOHANSSON, Oxygen uptake and tissue oxygen tension during sympathetic stimulation in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1976. 97. 39.
- HALLER, J. Sympathetic regulation of metabolism. *Pharmacol. Rev.* 1967. 19. 367-461.
- KEE, W. E., Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion systems, of glucose and of hyperventilation. *J. clin. Invest.* 1938. 37. 244.
- KEE, A. G. B., S. KOSILL, P. SANDOR, E. KOL, E. KOVÁČ and N. TOMKA, Blood flow, oxygen consumption, and free fatty acid release in subcutaneous adipose tissue during hemorrhagic shock in urea and phentolamine-treated dogs. *Circulat. Res.* 1970. 26. 773.
- KEE, A. G. B., E. KOVÁČ, P. SANDOR, J. A. SPITZER and J. J. SPITZER, Metabolic response to localized stress in adipose tissue. *J. Surg. Res.* 1976. 20. 57-64.
- KIL, S. and G. TIZELING, An enzymatic fluorimetric micro-method for the determination of glycerol. *chem. Acta* 1966. 13. 317.
- KIL, S. and G. TIZELING, Colorimetric micro-method of free fatty acids in plasma. *Chim. chim. Acta*. 57. 16. 57.
- KIL, S., Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol. scand.* 1966. 87. 343.
- KIL, S., P. SANDOR and A. G. B. KOVÁČ, Adipose tissue and hemorrhagic shock. In *Neurohumoral & Metabolic Aspects of Injury* (Eds. A. G. B. Kovács, H. B. Steiner and J. J. Spitzer.) Plenum Publ. NY. 1977. Pp. 323-336.

Discussion

The present results confirm the opinion that adipose tissue blood flow is of importance for the mobilization of lipolytic products, *i.e.* glycerol and FFA, from the tissue (Fredholm 1966, Fredholm 1970). A severe reduction of adipose tissue blood flow does not appear to impair the lipolytic response to sympathetic nerve stimulation, since the net release of glycerol was unchanged. On the other hand, the glycerol mobilization was delayed; a substantial part of the release occurred after the period of restricted flow. When flow is induced in a tissue with low blood flow glycerol may therefore be trapped in the tissue for very long periods of time before mobilization. This implies that observed interplasma glycerol not necessarily reflect the lipolytic rate in the adipose tissue at a given time. Himms-Hagen (1967) has enumerated several factors that tend to make conclusions concerning actions in adipose tissue from studies of plasma FFA and glycerol levels uncertain. The present findings seem to add yet another complicating factor to that list.

While blood flow restriction did not affect the total amount of glycerol released during a period of nerve stimulation (if sufficient time for sampling was allowed for) there was a reduction of the net release of FFA. This suggests that blood flow restriction has influenced the re-utilization of fatty acids in the tissue.

The increased utilization of FFA during blood flow restriction is best explained by an increased rate of reesterification of newly formed fatty acids. Several factors may contribute to this. Firstly a low blood flow and thus a decrease in carrier capacity (*i.e.* albumin) increase the concentration of FFA within the tissue. Secondly the increased lactate/pyruvate ratio indicates an elevated NADH/NAD⁺ ratio (Huckabee 1958). This will enhance the formation of α -glycerophosphate which is the other substrate required for triglyceride synthesis in the adipose tissue, as discussed previously (Fredholm 1971).

The present experiments show that the adipose tissue is not damaged by blood flow restriction during 60–90 min since stimulated glycerol and FFA-outflow as well as glucose uptake were almost identical during observation periods before and after blood flow restriction. Similarly Kováčik *et al.* (1976) found no irreversible damage to unstimulated adipose tissue when blood flow was reduced to 15% of control for 60 min by mechanical clamping. Thus restriction of adipose tissue blood flow to less than 20% of control does not give any evidence of ischemia.

These findings are in agreement with the findings that adipose tissue oxygen tension is only slightly reduced during mechanical clamping (Fredholm *et al.* 1976). However, noradrenaline, causing both active vasoconstriction and metabolic activation, lowers adipose tissue oxygen tension substantially (Fredholm *et al.* 1976). These differences may explain why experimental hemorrhage in contrast to a mere blood flow reduction does not cause irreversible damage to adipose tissue (Kováčik *et al.* 1970, Fredholm and Fredholm 1974) since hemorrhage is accompanied by intense sympatho-adrenal activation.

With these considerations as a background it may seem puzzling that blood flow restriction reduces adipose tissue oxygen uptake (Fredholm *et al.* 1976, Kováčik *et al.* 1976). We would like to propose that this decrease in oxygen uptake is caused by the simultaneous decrease in glucose uptake by the tissue, and hence by a decreased oxygen demand. A primary factor

adipose tissue is to store, as economically as possible, surplus energy delivered to it. It can be calculated that a major part of the substrate delivered to adipose tissue is used, not to subserve basic metabolic needs, but for synthesis, presumably triglycerides (Fredholm and Karlsson 1970, Fredholm *et al.* 1976). Thus, in this particular stressed energy supply (by for example reducing blood flow) may primarily lead not to compromised cellular function but merely to a reduced rate of energy storage.

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References

1. L.-O. B. B. FREDHOLM, B. HAMMARSTRÖM, P. HEDENRUBIN and L. WESTMAN, Cyclic AMP and catecholamines in hemorrhagic shock of the rat. *Acta chir. scand.* 1977 143, 9-14.
2. L. B. B., Studies on the sympathetic regulation of circulation and metabolism in isolated canine tumour adipose tissue. *Acta physiol. scand.* 1970, 80, Suppl. 354.
3. L. B. B. The effect of lactate in canine subcutaneous adipose tissue in situ. *Acta physiol. scand.* 87 110-123.
4. L. B. B. and J. KARLSSON, Metabolic effects of prolonged sympathetic nerve stimulation in canine tumour adipose tissue. *Acta physiol. scand.* 1970, 80, 567-576.
5. L. B. B. and A. FÄRBER, High energy phosphate compounds in adipose tissue: The effect of stress. *Acta physiol. scand.* 1974, 91, 163-171.
6. L. B. B., B. LINDH, R. L. PRUITT and P. C. JOHNSON, Oxygen uptake and tissue oxygen tension in adrenergic stimulation in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1976, 97, 9.
7. HADLEY, J., Sympathetic regulation of metabolism. *Pharmacol. Rev.* 1967, 19, 367-461.
8. H. W. E., Relationships of pyruvate and lactate during anaerobic metabolism. I. Effects of infusion of glucose and of hyperoxemia. *J. clin. Invest.* 1958, 37, 244.
9. A. G. B. S. ROSS, P. SÄNDORF, E. KÖRÖS, E. KOVÁČIK and N. TONELA, Blood flow, oxygen consumption, and free fatty acid release in subcutaneous adipose tissue during hemorrhagic shock in rat and phenylephrine-treated dogs. *Circulat. Res.* 1970, 26, 771.
10. A. G. B. S. KOVÁČIK, P. SÄNDORF, J. A. SPITZER and J. J. SPITZER, Metabolic responses to localized pain in adipose tissue. *J. Surg. Res.* 1976, 20, 37-44.
11. S. and G. THOMAS, An enzymatic fluorimetric micromethod for the determination of glycerol. *chem. Acta* 1966, 13, 317.
12. S. and G. THOMAS, Colorimetric micromethod of free fatty acids in plasma. *Chem. Abstr. Acta*, 7, 36, 57.
13. S. Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic stimulation. *Acta physiol. scand.* 1966, 87, 343.
14. S., P. SÄNDORF and A. G. B. KOVÁČIK, Adipose tissue and hemorrhagic shock. In *Neurochemical Aspects of Injury* (Eds. A. G. B. Kovács, H. B. Storer and J. J. Spitzer) Plenum Publ. 1971, pp. 323-336.

Potassium permeability of the mesothelium of the frog mesentery

By

J. FROKJÆR JENSEN and OVE CHRISTENSEN

Received 13 June 1978

Abstract

FROKJÆR JENSEN J and O CHRISTENSEN Potassium permeability of the mesothelium of frog mesentery. Acta physiol. scand. 1979 105: 228-238.

The mesothelium of the mesentery is a single cell layer described to offer no or very little hindrance to diffusion of small water-soluble solutes. We have measured the potassium permeability of the mesothelium of the frog mesentery both *in vivo* and *in vitro*. The permeability measured *in vivo*—using K⁺-sensitive microelectrodes is $2.4 \cdot 10^{-6}$ cm s⁻¹. *In vitro* measurements using conventional tracer flux techniques on isolated mesentery yield a K⁺-permeability of $5.7 \cdot 10^{-6}$ cm s⁻¹. These values are 15-30 times smaller than values previously reported from *in vitro* experiments on rat and rabbit mesentery. Also, the permeability is 12-15 times lower than the K⁺-permeability of the capillary wall determined on single capillaries of the frog mesentery. In the frog mesentery the mesothelium thus represents an important diffusion barrier compared to the capillary wall. This may be critical in experiments where filtration and reabsorption of solutes of the capillaries are determined from measurement of fluid exchange across the capillary wall in response to application of hypertonic solutions on the surface of the mesentery.

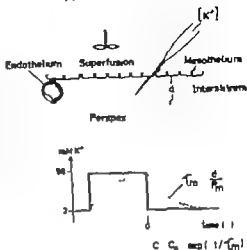
Many experimental studies of the filtration and diffusion properties of single capillaries have been performed on the capillaries of the frog mesentery (Landis and Sage 1971, Curry 1976, Crone *et al.* 1978, Curry 1978). The frog mesentery is a very suitable preparation since its microvessels here are larger than mammalian vessels and organized in a single layer in a thin sheet of interstitial tissue. The vessels are thus easily visible and accessible for experimental studies employing micro-tools.

The mesentery is covered by a single cell-layer—the mesothelium. The presence of this layer is usually neglected in experimental studies of filtration- and diffusion properties of the capillary wall to water and to small ions. This is well justified if the resistance of the mesothelium to movement of ions and water is much smaller than the resistance of the capillary wall. *In vitro* measurements of the mesothelial permeability in rat and rabbit have largely confirmed this assumption (Raslo 1974, Berndt and Cosselin 1962). However, studies of transmembrane ion transport through the autoperfused cat mesentery confirm the assumption (Fraser and Marcus 1976).

In this paper we report measurements of the permeability of the mesothelium of the frog mesentery to potassium ions. Measurements have been performed *in vivo* using

FROG MESENTERY

Schematic cross-section of the frog mesenteric mesentrium consists of an approx. 10 μ m thick layer containing few cells but a lot of folds. The mesentrium embeds the capillary walls are made up of endothelial cells. The mesentrium is covered towards the abdominal cavity by a single layer of cells forming mesothelium. The potassium concentration in mesentrium is measured by potassium-sensitive microelectrode. The lower half shows the 1/2 time behind the experiment. The superfusion (heavy line) is changed in square wave and the response in interstitial concentration (dashed line) is followed. The permeability is derived from the exposure-course of interstitial potassium concentration.



technique, and are supported by *in vivo* measurements, using a conventional flux technique.

The mesothelium of the frog mesentery is shown to be much less permeable than previously reported for rat and rabbit mesentery—but in accordance with values reported from cat mesentery. Recently we reported measurements of the potassium permeability of the capillary (Crosce *et al.* 1978). The present values of mesothelial permeability are 12–15% lower than the capillary permeability.

The purpose of this paper is to stress the importance of the mesothelium as a diffusion barrier which can not be neglected in experiments on capillary fluid and solute exchange in frog mesentery.

Methods

Animals. Experiments were carried out on *Rana temporaria* at room temperature (20–24°C). The animals had been kept at 4°C for varying periods up to 6 months. Anaesthesia was established by placing the frogs either in 5% urethane or 0.5 M LiCl (tricaine methanesulphonate, Sandoz) solution until the mouth reflexes stopped. The abdomen was opened ventrolaterally by electrocautery to maximize bleeding. The frog was placed on its back on a small table and the mesentery carefully spread out over a small perspex block it could be transilluminated and viewed through a Wild M3 stereo microscope. For positioning platinum-iridium microelectrodes and of perfusion pipettes, Leitz micromanipulators were used. The exposed mesentery was kept moist by a continuous superfusion with frog Ringer's solution.

Experimental technique

Below lies behind the measurements of the mesothelial permeability is shown in Fig. 1. A potassium-sensitive microelectrode is inserted into the mesentrium to measure the response in potassium concentration resulting from a sharp increase of potassium concentration in the superfusion solution. When the superfusion is raised abruptly from 2 mM to a value of 30 mM, the interstitial concentration will approach 30 mM in an exponential fashion. Alternatively if the potassium concentration in the superfusion solution is kept back to 2 mM again after the interstitial concentration has reached 30 mM, the interstitial concentration (C_i) will decline exponentially according to the relation

$$C_i = C_0 \exp(-t/\tau_m)$$

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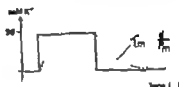
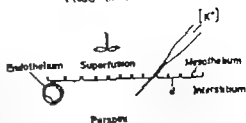


Figure 1

$$c_i = c_0 \exp(-t/\tau_m)$$

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Methods

Preparation

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$$c_i = c_0 \exp(-t/\tau_m)$$

(1)

where c_s is the superfusion concentration and where τ_m is the decay time constant. Concentration formulae are changes in concentration above the physiological concentration of 2 mM, such that c_{12} is 48 mM.

In this simple two-compartment system, where exchange of potassium takes place through the mesothelial surface the mesothelial permeability is given by the expression

$$P_m = d/\tau_m$$

where d is the thickness of the mesentery. A mathematical analysis of the system is given in the Appendix.

In practice it proved to be rather difficult to change the superfusion concentration abruptly disturbing the electrodes in position. Therefore another version of the experiment was adopted, in which the interstitium was loaded with a high potassium concentration from a single perfused capillary. A perfusion pipette connected to a manometer is filled with an isotonic frog Ringer's solution containing 2 mM potassium. A capillary is cannulated with the pipette and continuously perfused by applying pressure to the pipette. The superfusion concentration is maintained at 2 mM. Potassium ions permeate the capillary wall and diffuse into the interstitium where they permeate the mesothelium to be lost in the superfusion. After some time a steady state situation is reached (cf. Appendix). When steady state has been reached the perfusion is stopped and the decay of interstitial potassium concentration towards the superfusion concentration is monitored by a potassium-sensitive electrode placed in the interstitium. After a diffusional equilibration of interstitial potassium concentration the decay is monophasic and the determination of the decay time constant τ_m from which the permeability can be derived is straightforward. The change from the filling up situation to a discharge situation is simply done by turning the stopcock in the perfusion pipette without changing the flow of superfusion. In this way mechanical interference with measurements is avoided. Furthermore this method has the advantage of a steep concentration gradient across the mesothelium from the very beginning of the discharge phase.

In vitro control experiments

The mesentery of *Rana temporaria* was mounted in an Ussing chamber with an area of 0.33 cm². The mesentery was bathed in frog Ringer's solution at one side and samples were taken from the other side at 10 min intervals for 1 h. Unstirred layer effects were minimized by constant stirring on both sides by use of magnetic rods. Samples were counted by standard liquid scintillation technique and corrected for decay of the ⁴²K isotope. Several samples were taken from the 'hot' side during the experiment and counting of these showed that the ⁴²K-concentration on the 'hot' side remained constant during the experiment. Under these circumstances, the mesothelial permeability can be calculated from the equation

$$P_m = \frac{V}{S} \frac{d(\text{CPM})}{dt} \frac{1}{\text{CPM}}$$

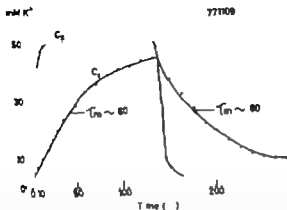
where V is the volume of one chamber (3 ml), S is the surface of mesentery (0.33 cm²), $d(\text{CPM})/dt$ is the rate of change of counts on the 'cold' side and CPM is the counts on the 'hot' side. The thickness of the unstirred layers can be estimated from the time course of change in membrane potential, where 30 mV is added to the medium on one side of the mesentery (Dalmy 1966). The total thickness of the unstirred layers was estimated to be about 160 μ m. The unstirred layers contribute thus to the calculated permeability with less than 5%.

The trans-mesenteric electrical resistance was measured at regular intervals during the experiment. The mean electrical resistance of the mesentery was $55 \Omega \text{ cm} \pm 15$ (S.D. $n=5$). Often a small decrease in resistance of 20–40% within 1 h after mounting the mesentery. This could indicate some minor deterioration with time due to the *in vitro* conditions. Contrary to this there was no indication of a systematic change in the potassium permeability in the flux experiments. The counts on the 'cold' side increased linearly with time.

Miscellaneous

Microtools: The preparation of the necessary microtools (perfusion pipettes, potassium sensitive and holding pipettes) and a description of the electrical set-up has been given elsewhere (Crosby *et al.* 1970). Each electrode was calibrated separately.

Isotopes. ⁴²KCl was obtained from AEK Riso, Denmark.



Experimental result showing the response of interstitial potassium concentration in response to an abrupt change in superfusate concentration from 2 mM K⁺ to 30 mM K⁺ and back again. c_o is the response recorded just at the surface of the mesothelium and c_i is the simultaneous response of an electrode less the mesothelium. The time constant for both the wash-in and the wash-out of interstitial concentration = 60 s.

user Normal frog Ringer' solution was prepared to provide the following concentrations (mM): 117 K, 2.0 Ca⁺⁺, 1.3 Cl⁻, 116.4 HCO₃, 1.2. The osmolality was 215 mosmol/L. The perfusate consisted of normal frog Ringer with increased K⁺-concentration, obtained by exchanging Na⁺

The potassium concentration was usually 30 mM. Bovine serum albumin (Sigma no. A-4303) was to final concentration of 1.25 g/L. The superfusate solution consisted of normal frog Ringer with urea solution added to concentration of 1.25 g/L. In experiments with increased K⁺-concentration Cl⁻ was exchanged for KCl. Calibration solutions were normal frog Ringer' solutions with NaCl replaced by KCl to give K⁺-concentrations of 2, 4, 8, 16, 25, 30, 75 and 100 mM.

Measurements of distance: The distances were measured by use of an eyepiece micrometer with an accuracy of 5 μ m.

Experimental results

Results

Fig. 2 gives an example of an experiment, where the mesothelial permeability was determined. The time course of interstitial potassium concentration (c_i) in response to an abrupt change in superfusate concentration (c_o) from 2 mM K⁺ to 30 mM K⁺ and back again. The concentration change in the superfusate is seen to be achieved within about 10 s while change in interstitial concentration is significantly retarded. The rise and decline of interstitial potassium concentration were characterised by exponential time courses with same time constant, from which the permeability was determined according to equation (2).

Fig. 3 gives an example of the other version of the experiment, where the interstitium is perfused with potassium from a continuously perfused capillary. When steady state was reached, the perfusion was stopped and the decay of interstitial potassium concentration was recorded. The decay at different distances from the capillary within the mesentery was obtained in consecutive runs. It is seen that after an initial time lag due to diffusional equilibration in the interstitium, the curves at all distances show a monoexponential decline. The deviation from the monoexponential time course is most marked close to the capillary

K^+ (mM)

40—

20—

10—

8—

6—

4—

2—

1—

13 μ m70 μ m128 μ m

Time (s)

Fig. 3. Wash-out of potassium from deer compartment. The decay of interstitial K^+ concentration is shown in various distances continuously perfused capillary through the interstitium has been loaded with potassium. curve starts when the perfusion is stopped. Curves are displaced for graphical reasons. Experimental points and continuous curve calculated according to the formula in the appendix: $D_s = 2.6 \cdot 10^{-6} \text{ cm}^2 \text{ s}^{-1}$, $P = 35 \cdot 10^{-3} \text{ cm}^2 \text{ s}^{-1}$, 65 s . The dashed line has a slope corresponding to decay time of 65 s . The experimentally determined decay time from the curve at $128 \mu\text{m}$ is 95 s .

where the initial concentration gradient is largest. On the figure is also shown a list of experimental data based on the theory given in the appendix. It is sufficient to see that it can be shown that a good approximation to the true time constant for wash-out of potassium can be obtained by determining the decay time constant from the slope at large times, i.e. at times comparable to the decay time constant. The mesothelial permeability was again determined from equation (2).

The results from experiments on 12 frogs is given in Table I. The mean thickness of interstitial compartment was found from 44 cross sections of frog mesenteries prepared by electron microscopy. An estimate of shrinkage of the tissue due to dehydration and bedding was made by comparing the dimension of *in vivo* red blood corpuscles with

TABLE I *In vivo* measurements of mesothelial decay time constants. The type of experiment is S for superfusion experiments and P for perfusion experiments. For the superfusion experiments, n is the number of determinations on the same position in the mesentery. For the perfusion experiments, n is the number of different positions from the perfused capillary used in the determination.

Type of experiment	Frog	Decay time constant (s)	n
S	1	45	3
S	2	60	2
S	3	54	1
P	4	51	3
P	5	47	3
P	6	44	3
P	7	32	3
P	8	48	2
P	9	41	2
P	10	38	2
P	11	66	2
P	12	93	4

Mean value 51.6 ± 16.0 (s.d.)

ones. A shrinkage of about 20% was found—a value in accordance with normally found shrinkage due to preparation for E.M. When a correction for a shrinkage of this kind was applied the mean thickness, d , of the frog mesentery was found to be

$$12.2 \pm 4.0 \mu\text{m} \quad (\text{s.d.}).$$

This figure is combined with the average decay time constant of 51.6 s (Table I) and mesothelial permeability P_m of

$$2.4 \cdot 10^{-8} \text{ cm s}^{-1}$$

calculated. It is not possible to give the standard deviation on this value since correcting values of thickness and decay time constant could not be determined on the same mesentery. The standard deviation on the decay time constant and the thickness are almost equal (31% and 33%, respectively). The standard deviation would thus vary from about 10 to 30% depending upon the degree of correlation between the decay time constant and thickness of the mesentery.

in vitro results

Results from 5 control experiments using radioactive tracer technique on the *in vitro* preparation gave a value of the mesothelial permeability of

$$P_m = 5.7 \pm 1.4 (\text{s.d.}) \cdot 10^{-8} \text{ cm s}^{-1}$$

and expected a large discrepancy between mesothelial permeabilities based on *in vivo* and *in vitro* techniques due to the possible shedding of the mesothelium of the *in vitro* preparation reported by Teal *et al.* 1971. Since this is not the case we have deferred from a more extensive investigation of the *in vitro* permeability.

in vivo control experiments

Questions concerning the *in vivo* experiments may be asked. The first concerns the efficiency with which the superfusion solution clears the upper mesothelial surface of the mesentery for potassium in excess of the concentration in the superfusate of 2 mM K. Evaluation of the mesothelial permeability is for both experimental versions based on the assumption of a superfusion concentration of 2 mM K.

In response to this question, the experiment on Fig. 4 illustrates the efficiency of the superfusion in clearing any excess potassium and shows qualitatively the importance of the abdominal wall as diffusion barrier.

A single capillary is cannulated with a perfusion pipette through which the capillary is loosely perfused with a Ringer's solution containing 50 mM K. Further downstream a potassium-sensitive electrode is positioned at various locations indicated in the inset of Fig. 4. First the electrode is inserted close to the capillary in the interstitium and the perfusion is started (A). Within 3 s the interstitial concentration at this place reaches a value of about 17 mM K. The electrode is withdrawn to a position just above the mesothelium in the superfusate (B), inserted again (C) and withdrawn. In (D) the electrode is positioned at the top of the perfused capillary and inserted in the interstitium

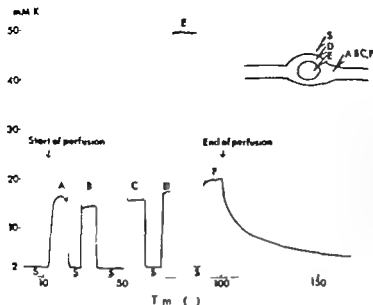


Fig. 4. Potassium concentration in capillary interstitium and capillary response to perfusion of 10% with 50 mM K⁺ free 1-2 solution. The K⁺ concentration in the superfusate is 2 mM. This sensitive microelectrode is at the positions shown in the figure. For further details refer to the text.

between the mesothelium and the capillary endothelium. A small tap at the micro-lator brings the electrode tip into the capillary lumen (E). In (F) the electrode is in the interstitium close to the capillary again and the perfusion is stopped.

The first part of the figure, S to A shows how rapidly K⁺ diffuses across the capillary endothelium in comparison with the rather slow exchange of potassium across the mesothelium (Fig. 2). The experiment stresses the importance of the mesothelium as a diffusion barrier which in the steady state situation, i.e. after 50–100 s holds a concentration of 2–20 mM K⁺ (S to F). The figure illustrates how efficient the superfusion is in keeping the K⁺ concentration constant at 2 mM K⁺ on the surface of the mesothelium, even after repuncture of both mesothelium and the capillary wall. Notice that the electrode response is immediately when it is moved from superfusate to interstitium and from interstitium to capillary which shows that there were no unstirred layers present.

The second question about the *in vivo* experiments concerns the possible existence of a fluid layer under the mesentery between the lower mesothelial layer and the surface of the perspex rod. Such a fluid layer would effectively increase the thickness of the mesentery, lead to a too large decay time constant and thus to a too small value of the mesentery permeability. Analysis of such a three-compartment system showed that if a fluid layer 10 μ m thick is present under a mesentery 10 μ m thick, the observed decay time constant would be larger by a factor of two than the true decay time constant. Consequently the calculated permeability would be underestimated by a factor of two.

In order to estimate the thickness of the possible fluid layer under the mesentery, series were consecutively mounted under a light microscope (Leitz Laborlux, objective 3210 40) with high magnification (320 times). At this magnification the focal depth is 5 μ m. The microscope was focused on a point in the tissue and a small glass rod (a No. 1 pipette) was used to press the tissue down towards the perspex rod. It was then depressed the tissue out of focus indicating the thickness of a layer. The calculated permeability is therefore

It is worth mentioning that 68% of diffusional equilibration over an interstitial distance of 10 μm takes place in less than 0.1 s, assuming a value for the diffusion constant D in the interstitium of $0.6 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Crooks *et al.* 1978). Interstitium can therefore be considered a well mixed compartment in the direction perpendicular to the surface of the mesentery.

Discussion

In vivo and the *in vitro*-results give upper and lower boundaries for the permeability of mesothelium. The *in vitro* results may be underestimated by maximally a factor of two because of possible existence of a fluid layer between the mesentery and the perspex rod. The results therefore represent a lower limit of permeability. Contrary to this, the *in vivo* results probably represent overestimates due to the *in vivo* conditions. The *in vitro* results therefore represent an upper limit of permeability. The true mesothelial permeability likely to be found within the range of these values, *i.e.*

$$2.5 \cdot 10^{-5} < P_m < 5.7 \cdot 10^{-5} \text{ cm s}^{-1}$$

Accuracy of the *in vivo* and *in vitro* results are not good enough to postulate a real difference between *in vivo*- and *in vitro*-permeability although the permeability measured *in vivo* is lower than the *in vitro* permeability.

Value for the mesothelial permeability can be compared with other published values. Mason and Berndt (1962) determined the Rb-permeability of the *in vitro* rabbit mesentery as $10^{-4} \text{ cm s}^{-1}$. The Rb-ion has approximately the same diffusion coefficient in aqueous solutions as the potassium ion. Rasio (1974) reported values of $79 \cdot 10^{-4} \text{ cm s}^{-1}$ for potassium permeability of the rat mesentery *in vitro*. Frasher and Marcus (1976) found a potassium permeability of $6.4 \cdot 10^{-5} \text{ cm s}^{-1}$ for the auto-perfused cat mesentery. Frasher (1978) has suggested, that albumin could affect the mesothelial permeability. In this series of *in vitro* experiments, we tested this hypothesis by measuring the transmembrane electrical resistance. The presence of albumin in the Ringer's solution had no effect on the transmembrane resistance.

The value of mesothelial permeability reported here is 12–25 times smaller than the potassium permeability of the capillary wall of $67 \cdot 10^{-5} \text{ cm s}^{-1}$ (Crooks *et al.* 1978). If this represents a poor model of the capillary wall, in contrast to the proposal by Rasio (1974), we conclude, that in the frog, the mesothelium of the mesentery differs significantly in its physical properties from the endothelium of the capillary. It represents an important diffusion barrier—a fact which may be crucial for the interpretation of experiments on fluid movement across the capillary wall induced by application of hypertonic solutions on the surface of the mesentery (Landis and Sago 1971; Curry *et al.* 1976; Curry 1978). The measured filtration of water across the capillary wall in these experiments is a function of both capillary and mesothelial permeability and of geometrical factors as well.

The authors thank professor C. Rose for his continuous support during the course of this work. The authors are also indebted to Dr M. Baudouin for valuable help and laboratory facilities for the *in vitro* experiments. The French Association is thanked for financial support.

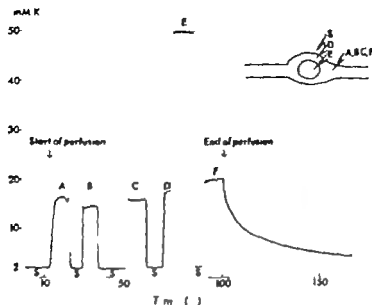


Fig. 4. Potassium concentration in different positions in response to perfusion of a capillary with 50 mM K-free solution. The K^+ -concentration in the superfusate is 2 mM. The electrode is positioned in the positions shown in the figure. For details refer to the text.

between the mesothelium and the capillary endothelium. A small tap at the micropipette brings the electrode tip into the capillary lumen (E). In (F) the electrode is in the interstitium close to the capillary again and the perfusion is stopped.

The first part of the figure, S to A, shows how rapidly K^+ diffuses across the endothelium in comparison with the rather slow exchange of potassium across the mesothelium (Fig. 2). The experiment stresses the importance of the mesothelium as a barrier which in the steady state situation, i.e. after 50–100 s holds a concentration of 2–20 mM K^+ (S to F). The figure illustrates how efficient the superfusion is in keeping the K^+ -concentration constant at 2 mM K^+ on the surface of the mesothelium, in spite of the repuncture of both mesothelium and the capillary wall. Notice that the electrode is immediately when it is moved from superfusate to interstitium and from interstitium to capillary which shows that there were no unstirred layers present.

The second question about the *in vivo* experiments concerns the possible existence of a fluid layer under the mesentery between the lower mesothelial layer and the surface of the perspex rod. Such a fluid layer would effectively increase the thickness of the mesentery, lead to a too large decay time constant and thus to a too small value of the mesenteric permeability. Analysis of such a three-compartment system showed that if a fluid layer 5 μ m thick is present under a mesentery 10 μ m thick, the observed decay time constant would be larger by a factor of two than the true decay time constant. Consequently the permeability would be underestimated by a factor of two.

In order to estimate the thickness of the possible fluid layer under the mesentery, three series were consecutively mounted under a light microscope (Leitz Laborlux, obj. 3210 40) with high magnification (320 times). At this magnification the focal depth is 5 μ m. The microscope was focused on a point in the tissue and a small glass rod (a pipette) was used to press the tissue down towards the perspex rod. It was not possible to depress the tissue out of focus indicating the thickness of a possible fluid layer to be less than 5 μ m. The calculated permeability is therefore underestimated by less than a factor

It is worth mentioning that 68% of diffusional equilibration over an interstitial distance of 10 μm takes place in less than 0.1 s, assuming a value for the diffusion constant in the interstitium of $0.6 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Crone *et al.* 1978).

Mesothelium can therefore be considered a well mixed compartment in the direction axial to the surface of the mesentery.

Discussion

The *in vivo* and the *in vitro* results give upper and lower boundaries for the permeability of mesothelium. The *in vitro* results may be underestimated by maximally a factor of two due to the possible existence of a fluid layer between the mesentery and the perspex rod. The *in vivo* results therefore represent a lower limit of permeability. Contrary to this, the *in vitro* results probably represent overestimates due to the *in vitro* conditions. The *in vivo* results therefore represent an upper limit of permeability. The true mesothelial permeability is likely to be found within the range of these values, *i.e.*

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The accuracy of the *in vivo* and *in vitro* results are not good enough to postulate a real difference between *in vivo* and *in vitro* permeability although the permeability measured *in vivo* is higher than the *in vitro* permeability.

The value for the mesothelial permeability can be compared with other published values. Landis and Berndt (1962) determined the Rb-permeability of the *in vitro* rabbit mesentery as $10^{-5} \text{ cm}^2 \text{ s}^{-1}$. The Rb-ion has approximately the same diffusion coefficient in aqueous solutions as the potassium ion. Raso (1974) reported values of $79 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for the potassium permeability of the rat mesentery *in vitro*. Frasher and Marcus (1976) reported a potassium permeability of $6.4 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ for the auto-perfused cat mesentery. Curry (1978) has suggested, that albumin could affect the mesothelial permeability. In a series of *in vitro* experiments, we tested this hypothesis by measuring the transmembrane electrical resistance. The presence of albumin in the Ringer's solution had no effect on the transmembrane resistance.

The value of mesothelial permeability reported here is 12–25 times smaller than the potassium permeability of the capillary wall of $67 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ (Crone *et al.* 1978). It thus represents a poor model of the capillary wall, in contrast to the proposal by Raso (1974). We conclude, that in the frog, the mesothelium of the mesentery differs significantly in its physical properties from the endothelium of the capillary. It represents an important diffusion barrier—a fact which may be crucial for the interpretation of experiments on fluid movement across the capillary wall induced by application of hypertonic solutions on the surface of the mesentery (Landis and Sago 1971, Curry *et al.* 1976, Curry 1978). The measured filtration of water across the capillary wall in these experiments is a function of both capillary and mesothelial permeability and of geometrical factors as well.

The authors are grateful to Dr M. Bendale for his continuous support during the course of this work. The authors are also grateful to Dr M. Bendale for valuable help and laboratory facilities for the *in vivo* experiments. The British Heart Association is thanked for financial support.

Appendix

In this appendix we shall derive an expression for the time course of the decay of interstitial K^+ concentration from the steady state in the situation where the interstitium is loaded through a mesenteric perfusion of the capillary. When the capillary is perfused with a constant potassium concentration will penetrate the capillary wall and be transported out in the interstitium by diffusion. However, mesenteric is flushed by a superfusion solution with a potassium concentration equal to the perfusate potassium concentration, ions will escape through the upper mesothelial layer. As we have seen (Crone *et al.* 1978) this leads to a steady state situation characterised by a spatial variation in K^+ concentration given by

$$c_i(x) = c_i(0) \exp(-x/\lambda)$$

where $c_i(0)$ and λ are given by the expressions (cf. Table A 1)

$$\lambda = (D_i \tau_m)^{1/2} \quad \tau_m = d/P_m$$

$$c_i(0) = \frac{c}{1 + (D_i/\lambda P)}$$

This steady state concentration is sketched in Fig. A 1. Note that the capillary permeability is only entering in determining the value $c_i(0)$ of concentration at the capillary wall. The physiological K^+ concentration equal to the one in the superfusion solution, has been subtracted from the concentration given by Eq. (A1) since only the increase above this value is being dealt with.

In order to describe the decay of the concentration from the steady state when the perfusion is stopped some approximations are appropriate. We shall here neglect the contribution from the potassium residing in the capillary. The time scale relevant for the decay situation is about 50 s. We have estimated that equilibration of the concentration difference across the capillary wall takes place during this time so that in the present analysis, the presence of the capillary can be neglected. The potassium ions in the capillary are neglected because they will redistribute in a volume much larger than the interstitium since the characteristic length for the interstitial concentration is about 200 μ m. The effect of the time course of the decay from the steady state will therefore only be substantial close to the capillary.

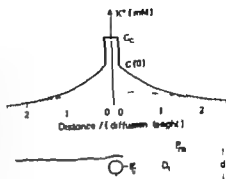
The change of concentration in the interstitium is described by the partial differential equation (Crone *et al.* 1978)

$$\frac{\partial c_i}{\partial t} = D_i \frac{\partial^2 c_i}{\partial x^2} - \frac{c_i}{\tau_m}$$

TABLE A 1 Symbols and units

Symbol	Unit	Significance
$c_i(x, t)$	mM	Interstitial potassium ion concentration.
c_c	mM	capillary concentration.
$c_i(0)$	mM	Interstitial concentration at the capillary wall.
d	cm	thickness of the mesentery
x	cm	distance from capillary wall to current position in interstitium.
λ	cm	characteristic diffusion length with the mesentery $(= (D_i \tau_m)^{1/2})$.
P	cm s ⁻¹	capillary permeability
P_m	—	mesothelial permeability
D_i	cm ² s ⁻¹	diffusion constant for potassium ions in interstitium.
τ_m	s	decay time constant for transport through mesothelium $(= d/P_m)$.

Sketch of potassium ion concentration in tissue versus distance normalized with respect to diffusion length. The full line shows the true concentration at time zero. The dashed line is the calculated concentration at time of the decay time constant τ_m . The lower half shows the position of the capillary in the tissue.



can be solved in space and time together with the boundary condition at zero time:

$$c_f(0, t) = c_f(0) \exp(-|x|/\lambda)$$

By symmetry of this curve around the origin we obtain as additional boundary condition, that the flux should be zero at the origin for times larger than zero

$$\left. \frac{\partial c_f}{\partial x} \right|_0 = 0 \text{ for } t > 0$$

Equations can be solved using the technique of Laplace transformation. The results for the decay of K^+ concentration from the steady state is:

$$c_f(x, t) = c_f(0) \exp(-|x|/\lambda) \left\{ 1 - \exp(-|x|/\lambda) \operatorname{erfc} \left(\frac{|x|}{\sqrt{2D}} \right) - \sqrt{\frac{2D}{\pi}} \exp(-|x|/\lambda) \operatorname{erfc} \left(\frac{|x|}{\sqrt{2D}} + \frac{1}{\tau_m} \right) \right\} \quad (A2)$$

A plot of this solution is shown in Fig. 3 at a distance of 0.46 τ_m after the end of perfusion. Because perfusion in the interstitial space is slow, concentration differences. When this spatial process has taken place in a given region of space, the decay of concentration in this region is purely exponential. This result can be obtained directly from Eq. (A2) when after sufficiently long a diffusion term can be neglected.

Fig. 3 shows a fit of τ_m to experimental data using the theoretical result in Eq. (A2). The values of λ and τ_m were first derived from the steady state results as described by Cross et al. 1978 and next changed to obtain the best fit.

Finally we have determined the value of τ_m from the decay of concentration far away from the capillary where diffusion effects are of minor importance. The decay here is a good approximation to an exponential. Nevertheless, this method tends to underestimate the value of τ_m as seen in Fig. 3. When to derive values for τ_m from a plot of the theoretical result (A2), using the slope of the decay at times equal to τ_m , the values thus derived are 10–15% smaller than the values used in the calculations. Hence, the experimentally determined value of τ_m should be underestimated by the same amount.

References

- C. J. FARMER, J. E. JENKIN, J. J. FREEDMAN and G. CHRISTENSEN, The permeability of single capillaries to various ions. *J. gen. Physiol.* 1978. 71: 193–220.
- F. L., Permeability coefficients of the capillary wall to low molecular weight hydrophilic solutes in single perfused capillaries of frog mesentery. *Microvasc. Res.* 1978. (in press)
- F. L., I. S. MASON and C. C. MICHEL, Osmotic reflection coefficients of capillary walls to low molecular weight hydrophilic solutes measured in single perfused capillaries of the frog mesentery. *Physiol. (Lond.)* 1976. 261: 319–334.

- DAINTY J and C. R. HOUSE, Unstirred layers in frog skin. *J. Physiol. (Lond)* 1966. 122: 6-7.
- FRASIER, W. G. and C. MARCUS, Transmembrane ion transport in autoperfused cat mesentery. *circulation*, Vol. 2, p. 86-87 1976. J. GRAYSON and W. ZINOC (Editt.), Plenum Press, New York.
- GOSSSELIN, R. E. and W. O. BERNDT, Diffusional transport of solutes through mesentery and μ . *J. Theoret. Biol.* 1962. 3: 487-495.
- LANDIS, E. M. and L. F. SAGE, Fluid movement rates through walls of single capillaries exposed to tonic solution. *Amer. J. Physiol.* 1971. 221: 520-534.
- RASO, E. M. Metabolic control of permeability in isolated mesentery. *Amer. J. Physiol.* 1974. 226: 123-128.
- TELL, D. P. DETRAZ and W. G. FOREMAN, Permeability studies with the intestinal tissue of the cat. *Pflügers Archiv ges. Physiol.* 1971. 332: 183-196.

Lack of specific prostaglandin antagonistic effects of 7-oxa-13-prostynoic acid on ovarian metabolism *in vitro*

4

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Abstract

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prostaglandins (PGs) have been proposed to function as obligatory intermediates in the action of luteinizing hormone (LH) (Kachi *et al.* 1970). This was based on experiments on isolated mouse ovaries where the mouse 7- α -13-prostaglandic acid (7- α -13-PA) was found to block the effect of both PGE₂ and LH on cyclic AMP formation and progesterone synthesis. We have studied whether 7- α -13-PA acts as a PG antagonist also on the isolated rat ovary where PGs of the E type have many effects. Prepubertal ovaries were incubated up to 4 h in modified Krebs bicarbonate buffer containing glucose with PGE₂, 17- α -13-PA, alone and in combination. PGE₂ stimulated the uptake of the non-oxidizable amino acids 1-methylhistidine (ASH) and cycloleucine in an equally wide dose-range as has earlier been found for stimulation of lactic acid and cyclic AMP production as well as protein synthesis. 7- α -13-PA alone, in concentrations exceeding 25 μ g/ml, stimulated lactic acid production, but inhibited the incorporation of methionine, exceeding 25 μ g/ml, stimulated lactic acid production, but inhibited the incorporation of methionine and the uptake of ASH. The uptake of cycloleucine was slightly stimulated. 7- α -13-PA did not antagonize the PGE₂ effect on lactic acid production. 7- α -13-PA diminished the PGE₂ effect on uptake of amino acids and the incorporation into protein, but only in concentrations where it in itself stimulated the protein synthesis. These results show that 7- α -13-PA does not act as a PG-antagonist in the prepubertal rat ovary since it cannot block the effects of PGs without having marked inhibitory effects.

Active work is being done in order to elucidate the role of prostaglandins (PGs) in the mechanism of action of various hormones. For this purpose several types of inhibitors of the action of PGs have been developed (for ref. see Samner 1974). In the late sixties Friedl and co-workers synthesized a group of substances with close structural similarity to PGs (Friedl *et al.* 1969). Several of these 7-oxa-prostaglandin analogues inhibited PG-induced contractions of isolated intestinal smooth muscle preparations in a competitive manner. The most specific PG inhibitors were the analogues with a triple bond in the 13-14-position. The authors, however, did not get the same clear-cut results. Fleck (1970) considered 7- β -13-prostynoic acid (7-oxa-13-PA) as the only specific PG inhibitor among these analogues. In several other experimental systems even this substance had no inhibitory effect.

In 1971 Bennett and Posner reported that 7-oxa 13-PA did not specifically inhibit the K⁺-induced contractions of several different intestinal smooth muscle preparations. In spite of these contradictory findings, 7-oxa 13-PA has been widely used as a PG antagonist in several different experimental systems (see e.g. Sanner 1974).

PGs exert many different actions in the reproductive sphere. The E type has been shown to mimic many effects of gonadotropins on ovarian metabolism. Thus, in the ovary it stimulates steroid synthesis (Pharriss *et al.* 1968, Marsh 1970, Speroff and Ramoel 1972), formation of cyclic 3',5'-adenosine monophosphate (cAMP) (Kuehl *et al.* 1970, Marsh 1970, Åhrén *et al.* 1974, Selstam *et al.* 1974), glycolysis (Perklev and Åhrén 1971) and fat acid transport and incorporation into protein (Åhrén and Perklev 1973). It was therefore of great interest when Kuehl and co-workers (1970) reported that 7-oxa 13-PA (50–75 µg) inhibited not only the PG-induced cAMP formation in the mouse ovary but also the stimulatory effect of LH on the cAMP formation. In contrast to these findings no inhibition of the stimulatory effect of LH or PGs on cAMP formation was found in porcine granulosa cells (Kolena and Channing 1972) or rat ovaries (Lamprecht *et al.* 1973). Ellsworth and Arnesen (1974) could not find that 7-oxa 13-PA affected the luteinization of granulosa cells in transplanted rat ovarian follicles in response to LH or PGE₂. Channing (1972) could find no inhibition on luteinization of monkey granulosa cells but when added together with human chorionic gonadotrophin (HCG) or PGE₂, 7-oxa 13-PA produced necrotic changes in the cells.

In the light of these studies it was considered to be valuable to study more in detail whether 7-oxa 13-PA has PG-agonistic and/or PG-antagonistic action. The effects of 7-oxa 13-PA alone and in combination with a prostaglandin, on the carbohydrate and amino acid metabolism were studied in an experimental system often used in our laboratory, the preovulatory rat ovary. Some preliminary results have been reported earlier (Åhrén and Perklev 1974).

Materials and Methods

Animals

Rats, 23 days old, of the Sprague-Dawley strain, were used. A standard pellet diet was given *ad libitum*. The rats were obtained from Anticimex Ltd, Stockholm and deprived of food 18–24 h before the experiment.

Chemicals and hormones

Generally tritiated α -aminoisobutyric acid (AIB-H), AIB-1-¹⁴C, 1-aminocyclopentane-1-carboxylic acid (¹⁴C-cycloleucine-¹⁴C), generally labelled leucine-³H and leucine-¹⁴C, and tritiated cyclic 3',5'-adenosine monophosphate (³H-cAMP) were obtained from New England Nuclear Co., Boston, Mass., USA. 1-non-utilizable amino acids AIB and cycloleucine had a final concentration in the incubation medium 0.1 mM. AIB-H had an activity of 0.33–1.65 µCi/ml, AIB-¹⁴C 0.057–0.2 µCi/ml and cycloleucine-¹⁴C 0.025–0.1 µCi/ml. The labelled natural amino acids leucine-³H and leucine-¹⁴C were used in a final concentration of 0.01 mM and had a activity of 1.98 µCi/ml and 0.2 µCi/ml, respectively. cAMP dependent protein kinase and protein kinase inhibitor for the cAMP assay were obtained from Sigma Ltd. All chemicals were of analytical grade and purchased from Sigma Ltd and Merck Co.

PGE and PGE₂ were supplied by Ono Pharmaceutical Co. Ltd, Osaka, Japan and PGF_{2α} by Upjohn Co. The 7-oxa 13-PA was kindly given to us by Prof. J. Friedl, Dept. of Chemistry and Biochemistry, University of Chicago, Ill., USA. The PGs and the 7-oxa 13-PA were dissolved in ethanol (1 µl ethanol per µg and 0.2 µl per µg 7-oxa 13-PA). Aliquots of these solutions were added directly to the incubation medium. Because ethanol in concentrations exceeding 10 µg/ml incubation medium in itself significantly inhibits

and increased cyclo-oxygenase uptake, concentrations higher than 20 µg PGE₂/ml or 2-oxo-13-PA/ml were not tested. Bovine LH (NIH-LH-B6) was generously supplied by the Endocrinology Section of the National Institutes of Health, Bethesda, Md, USA.

total procedure

were killed by cervical fracture and the ovaries were rapidly removed and chilled in buffer. They were freed of extraneous tissues under microscope, rinsed, blotted on filter paper and each pair of ovaries placed in 10 ml Erlenmeyer flask. The ovaries were incubated at 37°C for 30-240 min in 1 ml Krebs-Ringer bicarbonate buffer (1.25 mM CaCl₂) pH 7.4 equilibrated with 95% O₂ + 5% CO₂, addition of 1 mg glucose/ml. The labelled substrates, the PGEs and 2-oxo-13-PA were added in defined concentrations as indicated in Results.

Incubation the ovaries were rinsed, weighed, and homogenized in 10% trichloroacetic acid (TCA) containing aliquots from the supernatant and incubation media were taken for radioactivity determination according to Nelson and Seibert (1974). The uptake of the labelled amino acids is given as the mean value at the end of the incubation period, calculated according to Abate and Robinson (1965). TCA precipitates were used for determination of the incorporation of radioactivity into proteins as described (Nelson and Seibert 1974) and expressed as DPM/µg protein. The protein content was measured according to Lowry et al. (1951).

Acid accumulation in the medium was determined at the end of the incubation period according to Seibert et al. (1963 a, b). The tissue content of cAMP was determined according to Gilman (1970). cAMP in the medium was determined by a modification of this procedure as has been described (Seibert et al. 1974).

Pool procedure

Values are given as standard error of the mean. All groups presented in figures or mentioned in the text are the mean of 5-15 pairs of ovaries. The values were compared by analysis of variance, followed by Newman-Keuls multiple range test for comparison between individual groups (Woolf 1966). A value of less than 0.05 was considered significant.

Effects of different prostaglandins and fatty acids on lactic acid production and amino acid uptake in preovulatory rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose. The lactic acid was measured at the end of the incubation period. The amino acid uptake is given as distribution ratio at the end of the incubation period.

substrate		Lactic acid production (µg/ml ovary)	AIB- ³ H distribution ratio (CPM/ml extracellular water) (CPM/ml medium)
1			
2			
3			
4			
5			
6			
7			
8			
9			
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is of significance vs. controls: -p < 0.01 N.S. not significant.

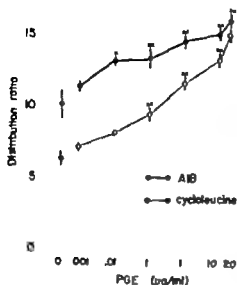


Fig. 1 Effect of PGE_2 on the uptake of AIB in prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose, 0.1 mM AIB, 0.1 mM cycloleucine- ^{14}C . The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE_2 effect: * $p < 0.05$, ** $p < 0.01$, N.S. = not significant.

Results

Effects of PGs

The effects of PGE , PGE_2 and $PGF_{2\alpha}$ on lactic acid production and uptake of AIB were compared (Table I). The potency of PGE and PGE_2 were equal, while $PGF_{2\alpha}$ was more potent. The uptake of cycloleucine was also measured after 2 h of incubation. PGE_2 and $PGF_{2\alpha}$ showed effects similar to those seen on the AIB-uptake (not shown in the Table). The PG precursor arachidonic acid and the fatty acid palmitic acid had no effect on any of the parameters (Table I).

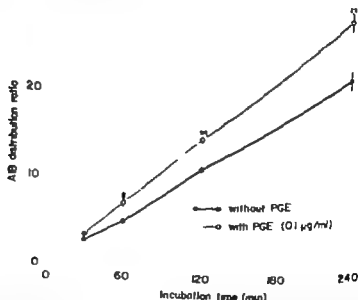
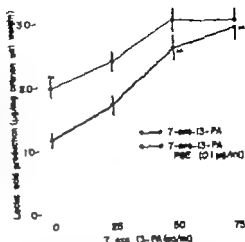


Fig. 2. Time-relationship of the uptake of AIB in prepubertal rat ovaries incubated with or without PGE_2 (0.1 µg/ml). The ovaries were incubated in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB- ^{14}C . The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE_2 effect at the different times: * $p < 0.05$, ** $p < 0.01$, N.S. = not significant.

Effect of 7- α -13-PA alone and in combination with PGE₂ (0.1 μ g/ml) on the lactic acid production by prepubertal rat ovaries. The ovaries incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose. Lactic acid was measured at the end of the incubation period. Levels of significance: * $p < 0.05$, ** $p < 0.01$, NS not significant. Levels of significance of the effect of the different concentrations of 7- α -13-PA alone are indicated at the lower curve. Levels of significance of the effect of PGE₂ at the various concentrations of 13-PA are indicated at the upper curve.



JE stimulates lactic acid production in a dose-dependent way in the concentration range 0.025–0.8 μ g/ml (Perklev and Abbrén 1971). In the present study we found that PGE₂ concentrations exceeding 0.1 μ g/ml did not give any further stimulation of the lactic acid production (data not shown). PGE₂ also stimulated the uptake of the non-utilizable amino acid AIB and cycloleucine in the ovary in the same range of concentrations that stimulated lactic acid production (Fig. 1). The dose-relationship of the effect of PGE₂ (0.1 μ g/ml) on AIB-uptake is shown in Fig. 2. The uptake is linear up to 4 h with time both with and without PGE₂.

In control ovaries, the incorporation of leucine-¹⁴C into ovarian protein after 2 h of incubation was 879 ± 22 DPM/ μ g protein and increased to 1078 ± 42 DPM/ μ g protein stimulated by 0.1 μ g PGE₂/ml medium. No further increase in incorporation rate was seen with higher PGE concentrations.

Effect of 7- α -13-PA

7- α -13-PA in concentrations ranging from 25 to 75 μ g/ml stimulated the lactic acid production in a dose-related manner (Fig. 3), i.e. had an effect similar to that of PGE₂. The effect of added PGE₂ was not abolished by 25 μ g 7- α -13-PA/ml. With higher concentrations of 7- α -13-PA, in itself strongly stimulating the lactic acid production, PGE₂ could not give further increase. In contrast to the effect on lactic acid production, 7- α -13-PA decreased the uptake of AIB, i.e. an effect opposite to that of PGE₂ (Fig. 4a). The effect of PGE₂ was not abolished by 7- α -13-PA. On cycloleucine-uptake, 7- α -13-PA had an inhibitory effect (Fig. 4b), but, as in the case of AIB-uptake, PGE₂ could stimulate the leucine-uptake when 7- α -13-PA was present.

The protein synthesis was inhibited by 7- α -13-PA in a dose-dependent way. Again PGE₂ had a stimulatory effect (Fig. 5), but only with 25 μ g 7- α -13-PA/ml where it in itself did not decrease the protein synthesis. Since 7- α -13-PA inhibited the protein synthesis it was tested if it was a specific inhibitor of the protein synthesis in the ovary. Puromycin in a dose of 10 μ g/ml (incubation medium) which inhibits the incorporation of labelled natural amino acids at approx. 95% (Abbrén and Rubinstein 1965), decreased the lactic acid production

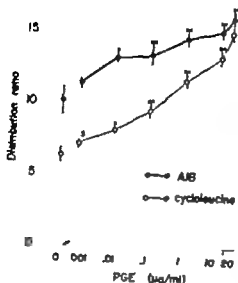


Fig. 1 Effect of PGE on the uptake of AIB and cycloleucine in prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer buffer containing 5.5 mM glucose, 0.1 mM AIB, 0.1 mM cycloleucine- ^{14}C . The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE effect: $p < 0.05$ — $p < 0.01$ N.S. = not significant.

Effects of PGs

The effects of PGE, PGE $_2$ and PGF $_{2\alpha}$ on lactic acid production and uptake of AIB are compared (Table I). The potency of PGE $_1$ and PGE $_2$ were equal while PGF $_{2\alpha}$ was less potent. The uptake of cycloleucine was also measured after 2 h of incubation. PGE $_1$, PGE $_2$ and PGF $_{2\alpha}$ showed effects similar to those on the AIB-uptake (not shown in the Table). The PG precursor arachidonic acid and the fatty acid palmitic acid had no significant effect on any of the parameters (Table I).

Results

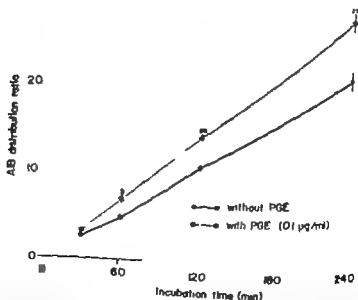
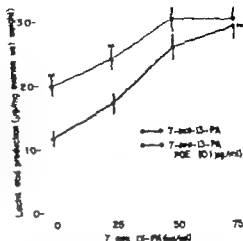


Fig. 2 Time-relationship of the uptake of AIB in prepubertal rat ovaries incubated with or without PGE (0.1 µg/ml). The ovaries were incubated in modified Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB- ^{14}C or AIB-H. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance of the PGE effect at the different times: $p < 0.05$ — $p < 0.01$ N.S. = not significant.

Effect of 7-oxa-13-PA alone and in combination with PGE_2 (0.1 $\mu\text{g}/\text{ml}$) on the lactic acid release by preperitoneal rat ovaries. The ovaries incubated for 2 h in modified Krebs-Ringer airtight buffer containing 5.5 mM glucose. Lactic acid was measured at the end of the incubation period. Levels of significance: * $p < 0.01$, NS - not significant. Levels of effect of 7-oxa-13-PA alone are indicated at the lower curve. Levels of significance of the effect of PGE_2 at the various concentrations of 13-PA are indicated at the upper curve.



E stimulates lactic acid production in a dose-dependent way in the concentration 0.025–0.8 $\mu\text{g}/\text{ml}$ (Perklev and Abrén 1971). In the present study we found that PGE_2 concentrations exceeding 0.8 $\mu\text{g}/\text{ml}$ did not give any further stimulation of the lactic acid action (data not shown). PGE_2 also stimulated the uptake of the non-utilizable amino AIB and cycloleucine in the ovary in the same range of concentrations that stimulated lactic acid production (Fig. 1). The time-relationship of the effect of PGE_2 (0.1 $\mu\text{g}/\text{ml}$) on AIB-uptake is shown in Fig. 2. The uptake is linear up to 4 h with time both with and without PGE_2 .

In control ovaries, the incorporation of leucine- ^{14}C into ovarian proteins after 2 h of action was 879 ± 22 DPM/ μg protein and increased to 1078 ± 42 DPM/ μg protein stimulated by 0.1 μg PGE_2/ml medium. No further increase in incorporation rate was with higher PGE_2 concentrations.

Effect of 7-oxa-13-PA

7-oxa-13-PA in concentrations ranging from 25 to 75 $\mu\text{g}/\text{ml}$ stimulated the lactic acid production in a dose-related manner (Fig. 3). It had an effect similar to that of PGE_2 . The effect of added PGE_2 was not abolished by 25 μg 7-oxa-13-PA/ml. With higher concentrations of 7-oxa-13-PA, in itself strongly stimulating the lactic acid production, PGE_2 could not give further increase. In contrast to the effect on lactic acid production, 7-oxa-13-PA decreased the uptake of AIB, i.e. an effect opposite to that of PGE_2 (Fig. 4 a). The effect of added PGE_2 was not abolished by 7-oxa-13-PA. On cycloleucine-uptake, 7-oxa-13-PA had inhibitory effect (Fig. 4 b), but, as in the case of AIB-uptake, PGE_2 could stimulate the leucine-uptake when 7-oxa-13-PA was present.

The protein synthesis was inhibited by 7-oxa-13-PA in a dose-dependent way. Again PGE_2 had a stimulatory effect (Fig. 5), but only with 25 μg 7-oxa-13-PA/ml where it in itself did not decrease the protein synthesis. Since 7-oxa-13-PA inhibited the protein synthesis it was supposed to be a specific inhibitor of the protein synthesis in the ovary. Puromycin in a dose of 0.01 $\mu\text{g}/\text{ml}$ incubation medium which inhibits the incorporation of labelled natural amino acids approx. 95% (Abrén and Rubinstein 1969), decreased the lactic acid production

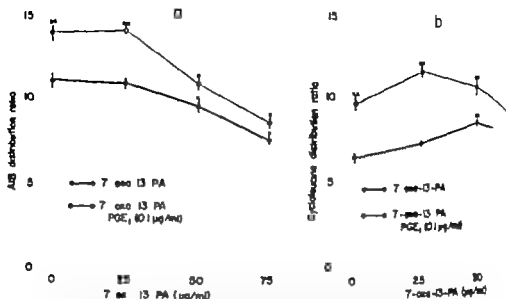


Fig. 4 a and b Effect of 7-oxa-13-PA alone and in combination with PGE₂ (0.1 µg/ml) on the AIB (a) and cycloleucine (b) in prepubertal rat ovaries. The ovaries were incubated for 2 h in a Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.1 mM AIB-³H or 0.1 mM cycloleucine-³H. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Levels of significance are indicated as in Fig. 3.

(control 16 ± 0.14 puromycin 0.34 ± 0.06 µg/ml w/w), somewhat decreased the AIB (control 8.5 ± 0.7 puromycin 6.4 ± 0.3) and increased cycloleucine uptake (control 4.1 puromycin 6.7 ± 0.3). Thus, on amino acid uptake and protein synthesis, 7-oxa-13-PA acted very similar to puromycin.

Due to the abovementioned marked inherent effects of 7-oxa-13-PA at concentrations above 25 µg/ml it was considered meaningful to test the effects of 7-oxa-13-PA on the cAMP system only at lower concentrations. 7-oxa-13-PA (1 and 20 µg/ml) did not change the cAMP content in the tissue nor the release of cAMP into the incubation medium (Table II). When 1 µg 7-oxa-13-PA/ml was added in combination with a gonadotropin

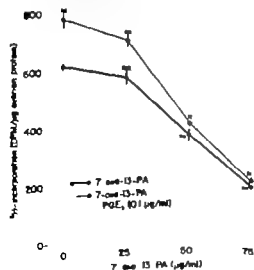


Fig. 5 Effect of 7-oxa-13-PA alone and in combination with PGE₂ (0.1 µg/ml) on the incorporation of radioactivity from leucine-³H into ovaries. Prepubertal rat ovaries were incubated for 2 h in a Krebs-Ringer bicarbonate buffer containing 5.5 mM glucose and 0.01 mM leucine-³H. Levels of significance are indicated as in Fig. 3.

influence of 7-oxa-13-PA on the stimulatory effect of LH on the cAMP content in the ovary and the incubation medium. The ovaries were incubated for 2 h in modified Krebs Ringer bicarbonate buffer containing 111 mM glucose.

1-PA cAMP (pmol/mg protein)

control		NIH-LH-B3 (1 µg/ml)	
time	medium	time	medium
15 ± 1	N.D.	72 ± 8	350 ± 120
14 ± 1 N.S.	N.D.	74 ± 10 N.S.	164 ± 32 N.S.
20 ± 2 N.S.	N.D.	166 ± 27 ^{***}	127 ± 16

N.D. = not detectable.

of significance of the effect of 7-oxa-13-PA. * $p < 0.05$ ** $p < 0.01$ N.S. = not significant.

ml), no effect was seen. At 20 µg/ml, however, an increase in the tissue content with a spontaneous decrease in the release of cAMP was found.

Discussion

of the important questions in the reproductive field is what roles the different PGs may play. When studying the function of PGs, several different experimental approaches have been used. Among these, we have further studied several gonadotropic effects that can be induced by the PGs, and we have also tested a proposed inhibitor 7-oxa-13-PA on these effects.

Gonadotropins stimulate amino acid uptake and lactic acid production (Hamberger and In 1967 Nilsson and Selstam 1974). In the present paper it was shown that a clear effect of a wide dose-response was achieved by PGE₂ on the uptake of the amino acids AIB and alanine, as well as production of lactic acid. Thus, on these parameters the ovary is as sensitive to PGs of the E type as it is on others, such as steroidogenesis and contractility. The effects of PGs of the F type observed in our study were much smaller.

Another way of testing the effects of PGs is by the addition of antagonists. One obvious criterion for an inhibitory substance is that the antagonist in inhibitory concentrations should have no or at least a less pronounced effect than the agonist, when competing for the receptor site. In this study the PG analogue 7-oxa-13-PA could not fulfil this criterion. It could only inhibit the PGE₂-effects in concentrations where it in itself had a marked stimulatory or inhibitory effect.

In this study we found no significant effect of 7-oxa-13-PA on amino acid transport and incorporation, or lactic acid production in concentrations up to 25 µg/ml. This result on amino acid transport is consistent with findings by Sato *et al.* (1972) in the thyroid.

In combination with LH, 7-oxa-13-PA exerted an effect on the cAMP system. The release of cAMP from the ovary was decreased and the tissue content increased. This redistribution may be due to a non-specific effect on the membrane, since in these concentrations 7-oxa-13-PA does not change adenylate cyclase activity as has been shown for many tissues by Iwabe *et al.* (1975).

The question arises whether the different effects of 7-oxa-13-PA are dependent upon each

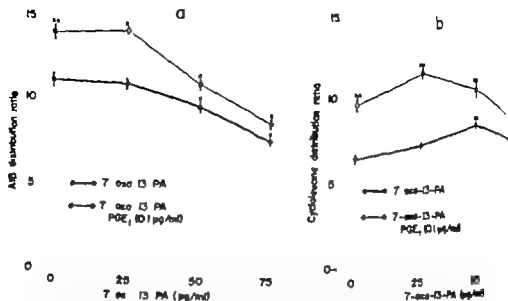


Fig. 4 a and b Effect of 7-oxa-13-PA alone and in combination with PGE₁ (0.1 µg/ml) on the uptake of AIB (a) and cycloleucine (b) in prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 3.5 mM glucose and 0.1 mM AIB-³H or 0.1 mM cycloleucine-³H. The uptake of amino acid is given as distribution ratio at the end of the incubation period. Line significance is indicated as in Fig. 3.

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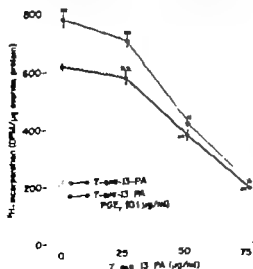


Fig. 5. Effect of 7-oxa-13-PA alone and in combination with PGE₁ (0.1 µg/ml) on the incorporation of radioactivity from leucine-³H into ovaries from prepubertal rat ovaries. The ovaries were incubated for 2 h in modified Krebs-Ringer bicarbonate buffer containing 3.5 mM glucose and 0.01 mM leucine-³H. Levels of incorporation are indicated as in Fig. 3.

1. Influence of 7-oxa-13-PA on the stimulatory effect of LH on the cAMP content in the ovary and the incubation media. The ovaries were incubated for 2 h in modified Krebs Ringer bicarbonate buffer containing 5.5 mM glucose.

1-PA cAMP (proteolytic proteins)

control		NIH-LH-B8 (1 μ g/ml)	
time	medium	time	medium
15 \pm 1	N.D.	72 \pm 8	350 \pm 120
14 \pm 1 N.S.	N.D.	74 \pm 10 N.S.	164 \pm 32 N.S.
20 \pm 2 N.S.	N.D.	166 \pm 27*	127 \pm 36

N.D. = not detectable.

* of significance of the effect of 7-oxa-13-PA: $-p < 0.05$, $-p = 0.01$ N.S. = not significant.

of), no effect was seen. At 20 μ g/ml, however, an increase in the tissue content with a spontaneous decrease in the release of cAMP was found.

Discussion

One of the important questions in the reproductive field is what roles the different PGs may play. When studying the function of PGs, several different experimental approaches have been used. Among these, we have further studied several gonadotropic effects that can be mediated by the PGs, and we have also tested a proposed inhibitor 7-oxa-13-PA on these systems.

Gonadotropins stimulate amino acid uptake and lactic acid production (Hamberger and Nilsson 1967 Nilsson and Selstam 1974). In the present paper it was shown that a clear effect on the amino acid dose-response was achieved by PGE₂ on the uptake of the amino acids AIB and glucose, as well as production of lactic acid. Thus, on these parameters the ovary is as sensitive to PGs of the E type as it is on others, such as steroidogenesis and contractility. The effects of PGs of the F type observed in our study were much smaller.

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The question arises whether the different effects of 7-oxa-13-PA are dependent upon each

other e.g. are the effects secondary to a general impairment of membrane function or the inhibition seen in protein synthesis? As for the latter suggestion, inhibitors of synthesis can inhibit AIB transport (Åhrén and Rubinstein 1965) and stimulate ox transport (Åhrén, Hillensjö and Selstam, unpublished), both findings confirmed in this study. It is therefore quite possible that the similar effects of 7-oxa-13-PA on amino acids might be secondary to the inhibited protein synthesis. On the other hand a change in membrane function may explain the change in amino acid transport as well as the increase in cAMP release and could perhaps also lead to an increased demand for energy as indicated by the increased lactic acid production.

In order to study the role of PGs in the ovary however it is also necessary to know whether PG-content and synthesis are changed after gonadotropic stimulation. In the rat and the ovary an increase of PGs has been shown after LH stimulation, but this increase is registered after an increase in cAMP content (LeMaire *et al.* 1973, Marsh *et al.* 1973, *et al.* 1974). Inhibitors of PG synthetase, such as indomethacin and aspirin, are ineffective in inhibiting the LH effect on adenylate cyclase as discussed by Marsh (1975).

Another mode of procedure when testing a possible role for PGs in the gonadotropic action is to study whether there is an additivity or not between the PG and the gonadotropin. Additivity would speak against a role of PG as an intermediate step in the gonadotropin action, while a lack of additivity is an indication, but not evidence of a role of PG. Moreover in the ovary additivity studies are complicated by the many different cell types which at least in part can be responsible for the different time-courses in cAMP release after LH and PGE₁ stimulation (Selstam *et al.* 1974).

In view of the findings that PGs mimic many of the actions of gonadotropins and that PG effects are related to changes in the cAMP system, it has been tempting to postulate a functional role for PGs in the ovarian metabolism. Inhibitors of PG action are one important tool for studying this role. Our conclusion from the present study however is that 7-oxa-13-PA is not appropriate in this respect, since it does not act as a PG inhibitor in the ovary.

The authors wish to thank Prof J. Fried, Department of Chemistry and Biochemistry, University of Chicago, Ill., USA, for the gift of 7-oxa-13-PA and valuable suggestions. Thanks are also due to Dr L. Helsingborg, Sweden, for the supply of PGs and to NIH for the supply of LH. The assistance of Stina Öberg, B.A., is gratefully acknowledged. This work was supported by grants from the Swedish Medical Research Council (B77 14X 27), Magnus Bergvall's Foundation, Wilhelm and Martina Lundgren's Foundation and from the Faculty of Medicine Göteborg.

References

- ÅHRÉN, K. and T. PERKLEV. Effects of PGE₁ and 7-oxa-13-prostanoic acid on the isolated preovulatory ovary. *Acta Obstet. gynecol.* 1973, 52, 717-721.
- ÅHRÉN, K. and L. RUBINSTEIN. Effects of follicle stimulating hormone on amino acid transport and protein synthesis in the isolated rat ovary. *Acta physiol. scand.* 1965, 64, 463-474.
- ÅHRÉN, K., H. HERLITZ, L. NILSSON, T. PERKLEV, S. ROSSBERG and G. SELSTAM. Gonadotropins and cAMP in various compartments of the rat ovary. In: *Gonadotropins and Gonadal Function*, ed. by C. Moodgal, Academic Press New York 1974, pp. 364-375.
- BENNETT, A. and J. PORMER. Studies on prostaglandin antagonists. *Brit. J. Pharmacol.* 1971, 42, 531-540.
- CHANNING, C. P. Effects of prostaglandin inhibitors, 7-oxa-13-prostanoic acid and ricin-3,5,6-tetrayonic acid, upon internalization of Rhesus monkey granulosa cells in culture. *Prostaglandins* 1977, 33, 331-337.

- ITA, L. R. and D. T. ARMSTRONG, Effect of indomethacin and 7-oxa-13-prostynoic acid on luteinizing hormone-stimulated rat ovarian follicles induced by luteinizing hormone and prostaglandin E_2 . *Prostaglandins* 1974, 7: 165-174.
- DA, D., Discussion in "Biological significance of the prostaglandins" *Rec Prog Horm. Res.* 1970 1: 179.
- T. S. SANTAKRISHNAN, J. HOSKIN, C. H. LIN, S. H. FORD, B. RUSBY and E. H. GRADAL, Prostaglandin antagonists: Synthesis and smooth muscle activity *Nature (Lond.)* 1969 223: 208-210.
- DA, A. G. A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. nat. Acad. Sci. (U.S.A.)* 1970, 67: 305-312.
- DA, L. A. and K. ARNIN, Effects of gonadotrophins h c r e on glucose uptake and lactic acid release of ovaries from prepubertal and hypophysectomized rats. *Endocrinology* 1967 81: 93-100.
- DA, J. CUPLIK, M. ČERNOMORECKÝ V KLIMKOVÁ, J. ŠKŘIVANOVÁ and M. WITKÝ, 7-oxa-13-prostynoic acid and polyphlorutin phosphates as non-specific antagonists of the stimulatory effects of different luteinizing hormone on adenylate cyclase from various tumours. *Prostaglandins* 1973, 10: 971-981.
- DA, J. and C. P. CHA, Stimulatory effects of LH, FSH and prostaglandins upon cyclic 3',5'-adenylate cyclase in porcine granulosa cells. *Endocrinology* 1972, 90: 1543-1550.
- DA, F. A. JR., J. L. HUMEL, J. TAKOVY V J. CIRILLO and E. A. HAN, Prostaglandin receptor interaction for an essential role in the action of luteinizing hormone. *Science* 1970, 169: 883-886.
- DA, S. A. U. ZOM, A. TAVAKOLI and H. R. LARSEN, Action of prostaglandin E_2 and of luteinizing hormone on ovarian adenylate cyclase, protein kinase and ornithine decarboxylase activity during postnatal development and maturity in the rat. *J. Endocr.* 1973, 37: 217-233.
- DA, W. J. N. S. T. Y. and H. R. BRIDGMAN and J. M. MARSH, Pre-ovulatory changes in the concentration of prostaglandins in rabbit Graafian follicles. *Prostaglandins* 1973, 10: 367-376.
- DA, O. H. N. J. ROBINSON, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin method. *J. Biol. Chem.* 1951, 193: 265-275.
- DA, L. E. MORRIS-LUDWIG and N. SVETKEY, Cooperative investigation of methods for determination of lactic acid in blood and in tissue extracts. *Small. J. Clin. Lab. Invest.* 1963 a, 15: 311-316.
- DA, L. E. MORRIS-LUDWIG and N. VANOR, Lactic acid assay with L(+)-lactic acid dehydrogenase from rabbit muscle. *Acta physiol. acad.* 1963 b, 58: 243-249.
- DA, J. M., The stimulatory effect of prostaglandin E_2 on adenyl cyclase in the bovine corpus luteum. *Life* 1970, 7: 283-286.
- DA, J. M. The role of cyclic AMP in gonadal function. In: *Advances in Cyclic Nucleotide Research* P. Greengard and G. A. Robison, Raven Press, New York 1973, 6: 137-199.
- DA, J. M. T. M. MALLS and W. J. LAMARCA, Preovulatory changes in the synthesis of cyclic AMP by the Graafian follicles. *Science (Amst.)* 1973, 304: 197-202.
- DA, Y. S. J. ZAMICKO and D. T. ARMSTRONG, Stimulation of prostaglandin F synthesis by luteinizing hormone in rabbit ovarian follicles grown in organ culture. *Life Sci.* 1974, 15: 1731-1734.
- DA, C. L. and D. BELTAN, Effects of LH and HCG on the metabolism of the prepubertal rat ovary: release of amino acid transport, protein synthesis, and glycolysis. *Acta Endocr. (Kbb.)* 1974, 78: 134.
- DA, L. Y. and K. ARNIN, Effects of prostaglandins, LH and polyphlorutin phosphates on the lactic acid production of the prepubertal rat ovary. *Life Sci.* 1971, 10: 1387-1393.
- DA, W. B. L. J. WYNGAARDEN and O. D. GUTHRIE, Biological interactions between prostaglandins and luteinizing hormone in the rat. *Gonadotropin* ed. E. Rosenberg, Geron-X, Los Alamos, Calif 1968, pp. 1-129.
- DA, J. H. Substances that inhibit the actions of prostaglandins. *Arch. Int. Med.* 1974, 133: 133-146.
- DA, L. E. M. SZANO, K. KOWALEK and O. BURKE, Role of prostaglandin in thyrotropin action on thyroid. *Endocrinology* 1972, 90: 343-350.
- DA, T. G. J. L. K. VIT, S. ROSENBERG, L. GILBERT, T. PERKLEY and K. ARNIN, Comparison between the effect of luteinizing hormone and prostaglandin E_2 on ovarian cyclic AMP. *Prostaglandins* 1974, 11: 311.
- DA, L. Y. and P. RANVILL, Prostaglandin stimulation of $in vitro$ progesterone synthesis. *J. Clin. Endocr.* 1974, 30: 345-350.
- DA, C. M., *Principles of Biochemistry* 1st ed. D. van Nostrand Co. Inc. Princeton 1948.

In vitro release of intestinal dipeptidases from everted rings of rat jejunum

By

J. JOOL and T. LINDBERG

Glycyl-leucine dipeptidase (EC 3.4.13.2) and proline dipeptidase (EC 3.4.13.9), shortly localized in the cytoplasm of the enterocytes (Nörén *et al.* 1977), were quickly released from *in vitro* preparation of pig small intestinal mucosa (Josefsson and Sjöström 1976). About 50% of the total activity was released within 10-20 s from intact mucosa on slow magnet stirring. These dipeptidases were also easily released from similar preparation of rat jejunal mucosa *in vitro* (Lindberg *et al.* 1975, Silk and Kim 1976). This contrast situation *in vivo* only small amounts of glycyl-leucine dipeptidase activity was found in small intestinal content in man (Josefsson *et al.* 1968) and in rat (Josefsson and Lilja 1975). There was no difference between the *in vitro* and *in vivo* release of proline dipeptidase.

The present experiments were performed to determine the release of these dipeptidases from everted rings of rat jejunum into the medium, employing an experimental technique commonly used for *in vitro* absorption studies (cf. Wilson 1962).

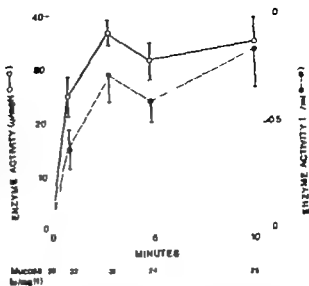
Materials. Rats (Sprague-Dawley strain), 200 to 250 g, fasted for 12 h in metabolic cages with free access to water. Under ether anaesthesia the small intestine was removed. Everted rings were prepared from jejunum (about 10 cm from duodenojejunal flexure). 6 rings were put in each incubation flask containing 5 ml of Krebs-Ringer phosphate saline gassed with O_2 and with 10 mmol/l glycyl-L-leucine (Biotec) and 2 mmol/l L-alanyl-L-proline (Sigma). Incubation was performed with shaking at 37°C under O_2 .

At the appropriate time, 1 ml of incubation medium was removed, cooled to 4°C (freezing does not destroy dipeptidase activity completely) and assayed immediately for dipeptidase activity according to Jool and Lindberg (1965). One of the everted rings was homogenized and analysed for dipeptidase activity. The enzyme assays were performed under optimum pH conditions (Josefsson and Lindberg 1966). One unit of dipeptidase is the activity hydrolysing 1 μ mol of dipeptide per min at 40°C.

Protein was determined according to Lowry *et al.* (1951) using crystallized bovine ribonuclease as standard. The nitrogen content was calculated as 17% of the protein content.

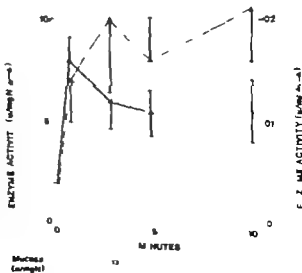
Results. Fig. 1 and 2 demonstrate the release of glycylleucine and of proline dipeptidase activities into the incubation medium. The activities are expressed as units per mg N₂ per ml of medium. The dipeptidase activities in the mucosa in the rings are given for comparison. Obviously both enzymes are quickly released; considerable activity is found after 1 min. The specific activity is as high as in the mucosa. 0.5 to 1.0 mmol/l glycyl-L-leucine and 0.1 to 0.2 mmol/l L-alanyl-L-proline are split per min by the enzymes released into incubation medium.

Glycyl-leucine dipeptidase (substrate glycyl-L-leucine) was during incubation with jejunal rings in the rat. The activity is expressed as μM (O—O) and U/mg (---). Mean values \pm S.E. Mean values for successful enzyme dipeptidase activity are for comparison.



results. The present study shows that a quick release of the cytoplasmic glycyl-leucine dipeptidase and proline dipeptidase occurs from the mucosa into the incubation medium in the *in vitro* experimental model of everted intestinal rings. Both this model and that of intestinal sacs are the most commonly used *in vitro* studies of the absorption of peptides and amino acids (cf Matthews 1975, Wiseman 1977). A considerable fraction of the enzyme applied to the incubation medium can be hydrolysed within minutes by the released enzymes. Glycyl-leucine dipeptidase has a wide specificity (Noren *et al.* 1973),

Proline dipeptidase activity (substrate L-alanyl-L-proline) was during incubation with jejunal rings in the rat. The activity is expressed as μM (—Δ) and U/mg (—Δ). Mean values \pm S.E. Mean values for successful enzyme dipeptidase activity are for comparison.



whereas proline dipeptidase splits dipeptides with the configuration of X-L-proline X-L-hydroxyproline (Sjöström *et al* 1973)

In view of these findings it is important to consider these properties of the cyclic intestinal dipeptidases when interpreting the results of dipeptide absorption *in vitro*

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References

- JONSSON, L. and T. LINDBERG, Intestinal dipeptidases I Spectrophotometric determination and characterization of dipeptidase activity in pig intestinal mucosa. *Biochim. biophys. Acta* (Amst.) 1972, 149-161.
- JONSSON, L. and T. LINDBERG, Intestinal dipeptidases III Characterization and determination of dipeptidase activity in adult rat intestinal mucosa. *Acta physiol. scand.* 1966, 66, 410-418.
- JONSSON, L., T. LINDBERG and L. ÖRSKÖLD, Intestinal dipeptidases. Dipeptidase activities in intestinal juice. *Scand. J. Gastroenterol.* 1968, 3, 207-210.
- JONSSON, L. and H. SJÖSTRÖM, Intestinal dipeptidases IV Studies on the release and subcellular localization of intestinal dipeptidases of the mucosal cells of the pig. *Acta physiol. scand.* 1966, 67, 77-83.
- LINDBERG, T., O. NORÉN and H. SJÖSTRÖM, Peptidases of the intestinal mucosa. In *Peptide and protein nutrition* (eds. D. M. Matthews and J. W. Payne). North-Holland, Amsterdam, 1973, 204-242.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with a phenol reagent. *J. biol. Chem.* 1951, 193, 262-275.
- MATTHEWS, D. H. Absorption of peptides by mammalian intestine. In *Peptide transport and nutrition* (eds. D. M. Matthews and J. W. Payne). North-Holland, Amsterdam, 1975, 99-114.
- NORÉN, O., E. DABELSTEIN, H. SJÖSTRÖM and L. JONSSON, Histological localization of two dipeptidases in the pig small intestine and liver using immunofluorescence. *Gastroenterology* 1977, 72, 57-62.
- NORÉN, O., H. SJÖSTRÖM and L. JONSSON, Studies on a soluble dipeptidase from pig intestine I Purification and specificity. *Biochim. biophys. Acta* (Amst.) 1973, 327, 446-456.
- SILK, D. H. A. and Y. S. KIM, Release of peptide hydrolases during incubation of intact intestinal cells. *J. Physiol. (Lond.)* 1976, 258, 489-497.
- SJÖSTRÖM, H., O. NORÉN and L. JONSSON, Purification and specificity of pig intestinal dipeptidase. *Biochim. biophys. Acta* (Amst.) 1973, 327, 457-470.
- WILSON, T. H., *Intestinal absorption*. W. B. Saunders Co. Philadelphia, London, 1962.
- WISMAN, G. Site of intestinal dipeptide hydrolysis. *J. Physiol. (Lond.)* 1977, 273, 731-743.

Control of the trunk during walking in the cat

By

HANS CARLSON, JÖNT HALBERTSMA and MICHAEL ZOMLEFER

There is a great deal of information concerning the electromyographic (EMG) patterns of the hindlimb in tetrapod locomotion (Griffioen 1975), there is little information about the control of the trunk. Recent experiments performed on the cat's back muscles have shown large portions of *M. longissimus* and *iliocostalis*, to be contracting and easily fatiguable, while the multifidi contract somewhat more slowly (1978 a, b). The aim of this study has been to explore patterns of activity in the lumbar back muscles during fast and slow walks and relate them to the trunk motion.

Our experiments were performed on cats (n = 3, 2.2, 2.3 and 3.2 kg), which were made to walk on a moving treadmill or overground at cycle durations ranging from about 1200 ms, corresponding to speeds ranging from 1.0 to 0.2 m/s, respectively. Bipolar needle electrodes were chronically implanted (Zomlefer *et al.* 1977) into the multifidi, longissimus (Lo) and iliocostalis muscles at various levels along the lumbar (L1-L6), as well as into both vasti laterales (VL). The EMG wires were led percutaneously to a plug on the animal's back, which could be connected with a detachable amplifier. Electrode placement was verified post mortem. The movements of one hind limb and the lower spine were recorded using a Schot System (Selcom Co. Partille, Sweden). Small light emitting diodes were glued onto the shaved skin over the spinal processes at Th9, L3, and L5 as well as the iliac crest and the hip, knee, ankle and phalangeal joints. Two cameras sampled each diode position at 160 Hz in both the sagittal and horizontal planes. Position and electromyographic data were fed online to a computer (Hewlett-Packard 21MX) for later processing.

The EMG activity in both the medially located multifidi muscles and the lateral longissimus and iliocostalis displayed two distinct large bursts during each step cycle (see Fig. 1A). The two bursts had similar durations and were synchronous in the different limbs, while each burst was tightly linked to the onset of the ipsi- and the contralateral swing (Fig. 1B). The duration of the bursts increased nearly threefold (from about 150 to 450 ms) whereas the amplitude (mean value of each burst per unit time) decreased by a factor of four as cycle duration increased over the range studied. There will thus be two periods of activity in the lumbar back muscles during each step cycle. These bursts will coincide with the periods of overlapping EMG activity in the limb extensor muscles of the sides, and thus they occur during the phases of double hindlimb support.

whereas proline dipeptidase splits dipeptides with the configuration of X-L-proline X-L-hydroxyproline (Sjöström *et al* 1973)

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References

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- JOERSSON, L., T. LINDBERG and L. ÖRSKÖLD, Intestinal dipeptidases. Dipeptidase activities in intestinal juice. *Scand. J. Gastroenterol.* 1968 3 207-210.
- JOERSSON, L. and H. SÖSTRÖM, Intestinal dipeptidases IV. Studies on the release and subcellular localization of intestinal dipeptidases of the mucosal cells of the pig. *Acta physiol. scand.* 1966 67 23-33.
- LINDBERG, T., O. NORÉN and H. SÖSTRÖM, Peptidases of the intestinal mucosa. In *Peptide and protein nutrition* (eds. D. M. Matthews and J. W. Payne). North-Holland, Amsterdam, 1971 704-740.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with a phenol reagent. *J. Biol. Chem.* 1951 193 62-75.
- MATTHEWS, D. M. Absorption of peptides by mammalian intestine. In *Peptide transport and nutrition* (eds. D. M. Matthews and J. W. Payne). North-Holland, Amsterdam, 1973 99-116.
- NORÉN, O., E. DÄBLSTEEN, H. SÖSTRÖM and L. JOERSSON, Histological localization of dipeptidase in the pig small intestine and liver using immunofluorescence. *Gastroenterology* 1977 72 111-118.
- NORÉN, O., H. SÖSTRÖM and L. JOERSSON, Studies on a soluble dipeptidase from pig intestinal mucosa. I. Purification and specificity. *Blackm. biophys. Acta (Amst.)* 1973 327 446-456.
- SILK, D. B. A. and Y. S. KIM, Release of peptide hydrolases during incubation of intact intestinal cells *in vitro*. *J. Physiol. (Lond.)* 1976 258 489-497.
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- WILSON, T. H. *Intestinal absorption*. W. B. Saunders Co Philadelphia, London, 1962.
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(1978 a, b). The aim of this study has been to explore patterns of activity in the lumbar back muscles during fast and slow walks and relate them to the trunk posture.

All experiments were performed on cats ($n = 3$ 2.2, 2.3 and 3.2 kg), which were trained to walk on a moving treadmill or overground at cycle durations ranging from about 1200 ms, corresponding to speeds ranging from 1.0 to 0.2 m/s, respectively. Bipolar stainless steel electrodes were chronically implanted (Zwiler *et al.* 1977) into the multifidus (M), longissimus (Lo) and iliocostalis muscles at various levels along the lumbar (L3-L6), as well as into both vasti laterales (VL). The EMG wires were led percutaneously to a plug on the animal's back, which could be connected with a detachable cable to amplifiers. Electrode placement was verified post mortem. The movements of one forelimb and the lower spine were recorded using a Selspot System (Selscom Co. Partille, Sweden). Small light emitting diodes were glued onto the shaved skin over the spinal vertebrae at T9, L3, and L5 as well as the iliac crest and the hip, knee, ankle and phalangeal joints. Two cameras sampled each diode position at 160 Hz in both the vertical and horizontal planes. Position and electromyographic data were fed online to a computer (Hewlett Packard 21MX) for later processing.

EMG activity in both the medially located multifidus muscles and the lateral longissimus and iliocostalis displayed two distinct large bursts during each step cycle (see Fig. 1A). The two bursts had similar durations and were synchronous in the different limbs, while each burst was tightly linked to the onset of the ipsi- and the contralateral swing (Fig. 1B). The duration of the bursts increased nearly threefold (from about 150 to 450 ms) whereas the amplitude (mean value of each burst per unit time) decreased by a factor of four as cycle duration increased over the range studied. There will thus be two distinct periods of activity in the lumbar back muscles during each step cycle. These bursts will coincide with the periods of overlapping EMG activity in the limb extensor muscles of the swing and stance phases, and thus they occur during the phases of double hindlimb support.

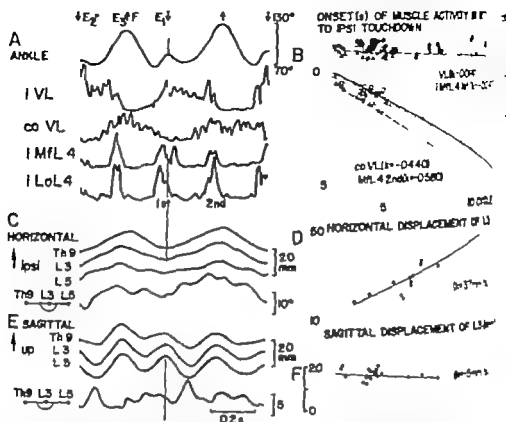


Fig. 1 A) Ankle angle (ANGLE) with rectified and filtered EMG's from ipsi- and contralateral VL as the ipsilateral Mf and Lo muscles at the L4 level (IVL, coVL, IMFL4 and ILoL4, respectively). The labeling, shown for the ipsilateral hindlimb, is the same as that used by Philippon (1905). In the EMG activity (relative to ipsilateral hindlimb touchdown) vs. cycle duration (cycle time) for VL and the first and second bursts of IMFL4 (IMFL41st and IMFL42nd). A positive value (+) indicates onset time is prior to ipsi-hindlimb touchdown. The time for onset of coVL will increase with cycle duration, since both hindlimbs are alternating in given step cycle. C) Horizontal displacement of L3 and L5 levels. The three uppermost curves show the relative trajectories of the marker nodes at the L3 and L5 levels. Movements toward the ipsilateral side (see A) is defined by arrow (+). The last shows the net angular displacement determined by L5-L3-Th9 (see inset). Minimum value corresponds to lateral bending with concavity toward the ipsilateral side. D) Peak to peak displacements in sagittal plane (L3 level) vs. step cycle duration. E) Sagittal displacements and angle. The first three traces show the movements of the three back diodes (Th9, L3 and L5 levels) as seen from the ipsilateral side. Movement upward is denoted by arrow (+). In the three cats investigated, systematic asymmetry in these traces, which may be due to (say) differences of the stiffness of the hindlimbs during the step cycle. Bottom trace shows the net angular displacements formed by 3 diodes as viewed in the sagittal plane (see inset), with minimum and maximum values corresponding to flexion and extension, respectively. F) Peak to peak displacements in sagittal plane (L3 level) vs. step cycle duration. The straight lines for the Mf and VL onsets ("—" and "—" respectively in B) as well as horizontal and sagittal displacements (in D and F) were constructed using the grouping method of Carlson (1949) with 3 groups (slope = k).

The movements in the spine will be a consequence of the amount of internal and external forces acting on the trunk. To estimate the intrinsic movements of the spine in the horizontal plane, the diode positions were compared with one another and the difference between L5-L3-Th9 is plotted (see lowermost curve in Fig. 1 C). It turns out that lateral bending lies in the range of 10-15°. However the movements of the spine (Fig. 1 C), i.e. the simultaneous movements of the three back markers, are small. The lateral movements decrease from about 40 mm at the L5 level to about 10 mm at the L3 level.

ness (Fig. 1 D). The body moves over to the side of the supporting hindlimb and is in the extreme lateral position at the end of the support phase. The trunk is thus moving from side to side with an amplitude that is maximal at low speeds. Apparently these movements are important to the equilibrium control of the animal and where most likely induced by limb action. In the sagittal plane, the intrinsic movements of the spine, flexions and extensions, are small, while the entire spine moves up and down about 1 cm during each step cycle (Fig. 1 E). These displacements are of about the same magnitude throughout a wide range of cycle durations (Fig. 1 F).

The EMG and movement data presented above suggest that the primary role of the muscles during walking is to control the stiffness of the back, rather than to induce movements.

This work was supported by the Swedish Medical Research Council (Project nr. 3026), and the Swedish Research Organization (Project nr. E1457), and funds from Karolinska Institutet. M. Z. holds a National exchange fellowship from the Swedish Medical Research Council. J. R. is supported, in part, by Delft University of Technology, the Netherlands.

The authors would like to thank Dr. Sten Grillner for his help in the preparation of this manuscript.

References

- LEVIT, M. S. Facing straight line: how both variables are subject to error. *Biometrics* 1949, 5, 1-112.
- ROTH, H. Morphology and contraction properties of cat lumbar back muscles. *Acta physiol. scand.* 1963, 89, 189-197.
- ROTH, H., Hatakenaka, fiber composition of lumbar back muscles in the cat. *Acta physiol. scand.* 1963, 89, 198-209.
- LEVIT, S. Locomotion in vertebrates—central mechanisms and reflex interaction. *Physiol. Rev.* 1973, 53, 7-394.
- PRIGON, M. Locomotion et la coordination dans le système nerveux des animaux. *Trav. Lab. physiol. Inst. Supér. Bruxelles* 1925, 7, 1-208.
- LEVIT, M. R., F. E. ZAJAC, and W. S. LAYMAN. Kinematic and muscular activity of cats during novel height jumps. *Brain Res.* 1977, 126, 3, 343-366.

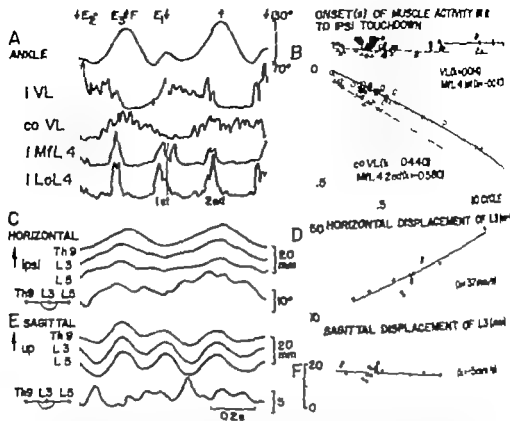


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References

- ATTI, M. S. Fitting a straight line when both variables are subject to error. *Biometrics* 1949, 5, 111-122.
- BOY, R. Morphology and contraction properties of cat lumbar back muscles. *Acta physiol. scand.* 1974, 92, 183-197.
- BOY, R. Histochemical fiber composition of lumbar back muscles in the cat. *Acta physiol. scand.* 1975, 92, 198-209.
- CLARK, S. Locomotion in vertebrates—central mechanisms and reflex interaction. *Physiol. Rev.* 1975, 55, 41-104.
- FERREY, M. L'automatisme et la centralisation dans le système nerveux des animaux. *Trav. Lab. physiol. Inst. Soler Bruxelles* 1905, 7, 1-208.
- FRUTKIN, M. R., F. E. ZALAC, and W. S. LEVINE. Kinematics and muscular activity of cats during vertical height jumps. *Brain Res.* 1977, 126, 2, 343-346.

Prostaglandin-mediated inhibition of noradrenaline release

VI On the intra-cardiac source of prostaglandins released from isolated rabbit hearts

By

ÅKE WENNMÄLM

Prostaglandins (PG) of the E , F and I series are released from isolated rabbit or pig hearts, as shown in studies using thin layer chromatography (TLC, Isaksson *et al.* 1977), gas chromatography (De Deckere *et al.* 1977), or bioassay (Schröer *et al.* 1978) for identification of the PG released. Although the physiological significance of cardiac PG has not been established, various hypotheses have been presented. Thus, PGI (prostaglandin) has been suggested to play a role in coronary vasodilation in response to hypoxia (cf. Neale and Kaley 1978), and PGE was recently proposed as a physiological modulator of sympathetic transmitter release in the heart (Wenmmalm 1978). Since these two different endogenous PG are exerted to some extent on different intra-cardiac structures (coronary vessels vs. sympathetic nerve terminals in the myocardium), we found it of interest to study whether PG formation in the heart is multifocal or alternatively is confined to one intra-cardiac tissue.

Rabbits of mixed strains and sexes, weighing from 2.0-3.0 kg, were used for the study. They were killed by a blow on the head and exsanguinated from the left carotid artery. The heart was rapidly removed and transferred to the perfusion apparatus, in which it was perfused according to Langendorff at a pressure of 60 cm H₂O with 37°C Tyrode solution. The solution was aerated with 5% CO₂ in O₂. Some hearts were, in addition, prepared according to De Deckere *et al.* (1977), which allows separate collection of coronary (Q_{cr}) and transmural (Q_t) effluent.

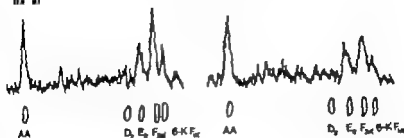
In one series of hearts, ¹⁴C-arachidonic acid (¹⁴C-AA, New England Nuclear, 40-60 mCi/mmol, 5 μ Ci), prepared as sodium salt, was infused during 15 min through a cannula close to the aorta under continuous collection of effluent. After the end of infusion of isotope, perfusion was switched to a non-aerated, 37°C Tyrode solution. Perfusion with this anoxic solution was maintained during 75-200 min. Following the anoxic period, perfusion was switched back to aerated Tyrode solution and a second infusion of ¹⁴C-AA was performed as above, with continuous collection of perfusate.

In another series the hearts after initial equilibration for 15-20 min in the perfusion apparatus, were infused with ¹⁴C-AA (10 μ Ci) during 30 min. Q_{cr} and Q_t were collected continuously during the entire infusion.

The cardiac effluents were immediately acidified to pH 4.5 and extracted with an equal vol. of ether. After washing of the pooled organic phases to neutral reaction

Fig. 1

Art hyp is (200 min)



Radioactive (thin layer chromatography) of cardiac effluents collected during infusion of ^{14}C -arachidonic acid. Left: effluent collected under basal conditions from spontaneously beating heart. Right: effluent collected after 200 min perfusion with an anoxic solution. The heart displayed no visible blood activity.

After anoxia, perfusion of the heart with ^{14}C -labelled sodium arachidonate yielded labelled PG in the effluent. The activity appeared chromatographically in two major peaks (apart from authentic ^{14}C -arachidonate), corresponding to $\text{PGF}_{2\alpha}$ and PGE_2 , and a smaller peak, corresponding to 6-keto- $\text{PGF}_{1\alpha}$ (Fig. 1). In most cases minor peaks were also observed, running parallel with PGD_2 and 13,14-dihydro-15-keto- PGE_2 (Me).

During the anoxic period the contractility of the myocardium rapidly faded, and after 20 min no visible mechanical activity of the organ occurred. Effluents collected during perfusion of ^{14}C -arachidonate after anoxia (maintained during 75, 90 and 200 min, respectively) displayed mainly the same chromatographic pattern of ^{14}C PG as effluents obtained during the anoxic period (Fig. 1), with respect to both the type and the amount of ^{14}C PG.

Separate collection of Q_{cor} and Q_{tr} during infusion of ^{14}C -arachidonate yielded the following data. Q_{tr} constituted 1.9–2.4 % of the total cardiac effluent, and contained 1.0–4.0 % of the infused isotope that was not retained in the organ. In the Q_{tr} effluents, $64 \pm 14\%$ (mean \pm S.E., $n = 3$) of the activity was identified as ^{14}C PG. In the Q_{cor} effluents the corresponding figure was $94 \pm 3\%$. The relative proportions of the various ^{14}C -PG-peaks in the Q_{cor} and the Q_{tr} effluents did not differ in any respect from each other (Table I).

Table I. Relative proportions of ^{14}C -PG in the coronary (Q_{cor}) and transmyocardial (Q_{tr}) effluents from 3 hearts infused with ^{14}C -arachidonate. The data are presented as mean \pm S.E. of the ^{14}C -PG identified in the respective chromatograms. Me stands for 13,14-dihydro-15-keto- PGE_2 .

	6-keto- $\text{PGF}_{1\alpha}$	$\text{PGF}_{2\alpha}$	PGE_2	PGD_2	Me
17	1	32 ± 3	26 ± 4	13 ± 3	11 ± 4
19	2	32 ± 3	29 ± 6	7 ± 1	13 ± 11

Comment

Sustained hypoxia (75–200 min) which completely annulled the contractility of the left ventricle, probably also much of the remaining metabolic activity of the myocardial cells, did not have any effect on the profile of ^{14}C PG in the effluent during infusion of ^{14}C -arachidic acid. This observation strongly suggests that the current effluent content of ^{14}C -PG was the result of enzymatic transformation of ^{14}C AA in the myocardial cells. Excluding the myocardium as a possible source of the cardiac PG formed from exogenous precursor draws attention to the alternative that the coronary vasculature was the origin of the PG system. This concept is supported by the experiments in which slow passage of ^{14}C -arachidic acid through the myocardium was not accompanied by a larger proportion of labelled material in the effluent than that found after rapid passage via the coronary vasculature, i.e. transmyocardial effluent (Q_1) did not differ in its ^{14}C PG profile from the coronary (Q_{cv}) effluent. In fact, the proportion of ^{14}C PG was smaller in the Q_1 (64%) compared to the Q_{cv} (94%) effluent.

In summary the present data strongly suggest that cardiac PG formation takes place mainly in the coronary vasculature. Since the adrenergic nerve terminals in the rabbit heart are distributed evenly throughout the tissue (Angelakos *et al.* 1963), it is unlikely that cardiac PG formation and sympathetic transmitter release are not strictly related morphologically. This statement leads one to question the universal validity of the hypothesis (Hedqvist 1970, Wennmalm 1971) that sympathetic transmitter release is subject to a negative feed back control mediated by endogenously formed PG.

This study was supported by the Swedish Medical Research Council (project 04X-4341). PG standards were kindly put at our disposal by Dr J. E. Pike at the Upjohn Company.

References

- ANGELAKOS, E. T. K. FUXE and M. L. TORCHIANA, Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea pig hearts. *Acta physiol. scand.* 1963. 59 (Suppl. 284).
- DE DECKERE, E. A. M. and H. NUOTTEREN and F. TEN HOOPE, Prostacyclin is the major prostanoid released from the isolated perfused rabbit and rat heart. *Nature (Lond.)* 1977. 263. 160–161.
- HEDQVIST, B. Studies on the effect of prostaglandins E_1 and E_2 on the sympathetic nervous system transmission in some animal tissues. *Acta physiol. scand.* 1970. 79 (Suppl. 345). 1–40.
- ISAACSON, P. C., A. RAZ, S. E. DENNEY, E. FUXE and P. NEEDLEMAN, A novel prostaglandin is the major product of arachidonic acid metabolism in rabbit heart. *Proc. nat. Acad. Sci. (Wash.)* 1977. 74. 661.
- NEEDLEMAN, P. and G. KALEY, Cardiac and coronary prostaglandin synthesis and function. *New Engl. J. Med.* 1978. 298. 1122–1128.
- SCHIRÖF, K., S. MONCADA, F. B. URAYURA and J. R. VANE, Transformation of arachidonic acid to prostaglandin endoperoxides by the guinea pig heart. Formation of RCS and prostacyclin. *Eur. J. Pharmacol.* 1978. 47. 103–114.
- WENNMALM, Å., Studies on mechanisms controlling the secretion of neurotransmitters in the rabbit heart. *Acta physiol. scand.* 1971. Suppl. 365.
- WENNMALM, Å., Prostaglandin-mediated inhibition of noradrenaline release: III. Separation of prostaglandins released from stimulated hearts and analysis of their neurosecretion inhibitory capacity. *Prostaglandins* 1978. 15. 113–121.

Uptake and release of adenosine in isolated rat fat cells

By

BERTIL B. FREDHOLM and PAUL HJERMDAHL

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Abstract

FREDHOLM, B. B. and HJERMDAHL, P. *Uptake and release of adenosine in isolated rat fat cells*. Acta physiol. scand. 1979 105: 257-267.

Radioactively labelled adenosine and adenosine were rapidly taken up by isolated rat fat cells, and incorporated into nucleotides, of which ATP dominated. The overall process had an apparent K_m of $1.5 \mu M$. During incubation, especially in the presence of lipolytic agents, there was a reduction in labelled ATP, a compensatory increase in ADP, AMP, cAMP and nucleosides. The build-up of adenosine during incubation was inhibited by theophylline, which inhibited 5'-nucleotidase. Radioactivity released from isolated fat cells consisted mainly of nucleoside material, of which adenosine predominated. Lipolytic stimulation caused no significant increase in adenosine outflow from perfused cells. Isolated erythrocytes capable of releasing this outflow. It is concluded that adenosine is formed by fat cells as a consequence of ATP breakdown. Stimulation of lipolysis during activation of the sympathetic nerves leads to reversible ATP breakdown and adenosine release. Adenosine might therefore act as a modulator of lipolysis *in vivo* in these conditions, even though it does not serve as a feed back regulator in the proper sense.

Adenosine has been ascribed a role as an endogenous modulator of cyclic AMP accumulation and lipolysis in adipose tissue (cf Fain 1973, Schwabe *et al.* 1975). Adenosine is formed by incubation of fat cells (Schwabe *et al.* 1973-1975), in perfused fat cells (Turpin *et al.* 1977) and in the *in situ* blood perfused canine subcutaneous adipose tissue (Fredholm 1976). Adenosine is a potent inhibitor of cyclic AMP accumulation in fat cells (Fain, Polster and *et al.* 1972, Fain 1973, Ebert and Schwabe 1973, Hjerm Dahl and Fredholm 1976). The deacetylase also inhibits lipolysis in isolated rat fat cells provided that the concentration of lipolytic stimulus to be antagonized is kept low (Fain 1973, Fredholm 1978) and provided that the level of endogenous adenosine is minimized either by using dilute cell suspensions (Ebert and Schwabe 1973) or by using a perfusion system (Turpin *et al.* 1977, Hjerm Dahl and Sollevi 1978). Adenosine is also a potent inhibitor of lipolysis induced by sympathetic nerve stimulation *in situ* (Fredholm and Sollevi 1977).

Addition of adenosine deaminase, an enzyme that converts adenosine to inactive inosine, increases lipolysis in isolated fat cells (Schwabe and Ebert 1974, Fain and Wieser 1975, Hjerm Dahl and Fredholm 1976). Moreover, dipyrindamol and dflazep, two inhibitors of adenosine uptake, which lack antilipolytic effects by themselves potentiate the antilipolytic effect of

adenosine *in vitro* (Fredholm 1978) and inhibit lipolysis induced by nerve stimulation *in vivo* (Fredholm and Sollevi 1977, 1978). These findings lend further support to the concept that endogenous adenosine might act as a modulator of lipolysis.

It was originally suggested that adenosine might act as a feedback regulator of lipolysis (Fain *et al.* 1972). In order to demonstrate such a feedback inhibitory role release of adenosine by lipolytic stimuli must be demonstrated. Schwabe and coworkers (1973) demonstrated accumulation of adenosine in the fat cell incubates but were unable to show a significantly enhanced accumulation in the presence of lipolytic hormones. Fain and Shepherd (1977) have been similarly unsuccessful in demonstrating a consistent effect of lipolytic drugs on adenosine release. Furthermore, in the intact perfused canine subcutaneous adipose tissue, sympathetic nerve stimulation induced release of adenosine-like material, which was inhibited by α -adrenoceptor antagonists which actually potentiate the lipolytic response (Fredholm 1976).

The aim of the present studies has been to further examine the question if lipolytic hormones release adenosine, and consequently if this nucleoside may be considered as a feedback regulator of lipolysis. Some of these findings were communicated in preliminary form at the meeting of the Scandinavian Physiological Societies in November 1977 (Huendahl *et al.* 1978).

Materials and methods

Epididymal fat pads from 2-5 decapitated Sprague-Dawley rats (Anticimex strain) weighing 130 g were pooled in each experiment. Isolated fat cells were prepared as described by Rodhe (1964), by reaction with 3 mg/ml crude bacterial collagenase (Worthington) for 40-60 min. The cells were washed three times before prelabelling or incubation. All incubations were carried out in Krebs-Ringer phosphate buffer pH 7.4 containing 5.5 mM glucose, 1.8 mM CaCl_2 and 3 per cent bovine serum albumin (fraction V, R1, Lohs).

Cyclic AMP was determined in the fat cell incubates with the protein binding assay described by Eriksson and Albano (1972) after treatment of the samples as described by Fredholm and Huendahl (1977). Thus, cells and medium were precipitated by perchloric acid (0.4 N final concentration). Following centrifugation the supernatant was neutralized by 4 N KOH and Tris base was added to a final concentration of 50 mM. The potassium perchlorate precipitate was removed by centrifugation after which cyclic AMP was determined on the supernatant.

Prelabelling of fat cells was performed by incubating a concentrated cell suspension for 18 min in 10-100 μCi [2- ^3H]-adenine (New England Nuclear, 23.0 Ci/mmol) or [8- ^{14}C]-adenine (43.95 $\mu\text{Ci/mmol}$) after which the cells were washed 3-6 times before subsequent incubations. In uptake experiments [4- ^{14}C]-adenine or [2,8- ^{14}C]-adenosine (New England Nuclear, 30.4 Ci/mmol) are added to a fat cell suspension at time 0. Aliquots of this suspension were subsequently removed at the indicated times and treated as described above for the determination of cyclic AMP.

The radioactive purities were separated by ion-exchange on thin layer chromatography. Group separation into nucleotide and non-nucleotide material was effected by chromatography on small (2.5 \times 87 cm) columns of Dowex 1-2 (200-400 mesh) essentially as described by Rutte *et al.* (1973). Separation of different nucleotides was performed either by chromatography on long anion-exchange columns as described previously (Fredholm *et al.* 1977) or by chromatography on PEI TLC sheets as described by Rutte and Schultz (1974). Nucleosides and bases were separated by thin-layer chromatography on silica gel as described by Pull and Mellwain (1972).

Perfusion experiments. Fat cells were isolated and prelabelled with ^3H -adenine as described above. After the final washing 1 ml of packed cells was transferred to a water-jacketed plastic chamber containing 2-2.5 ml Krebs-Ringer phosphate buffer pH 7.4 containing 1.8 mM CaCl_2 , 5.5 mM glucose and 1 per cent serum albumin. The floating fat cells were subsequently 'perfused' (Allen *et al.* 1973) with the buffer at 37°C and a rate of 2 ml/min. Noradrenaline or isoprenaline were diluted in saline containing 20 μg of

acid and balanced via side arm before the perfusion chamber. With the present technique steady concentrations of drug is obtained. After 5 min of infusion and lipolysis, measured as glycerol product, approaches steady state levels after 15–20 min of stimulation (Hjemsdahl 1976). ^3H -radioactivity in perfusion effluent was characterized and measured as described above.

Adenosine (EC 3.1.3.5) was determined according to Gentry and Olson (1975) as described by Solin *et al.* (1978). Fat cells from two rats are homogenized in 5 ml 60 mM Tris-acetate pH 7.4, centrifugation at $1400 \times g$ for 10 min the fat free supernatant was removed and diluted 5 times with same buffer. 0.05 ml of the extract was incubated for 15 min at 37°C with ^3H -5'-adenosine monophosphate (1–2 300 μM final concentration) in total volume of 1 ml 60 mM Tris-acetate pH 7.4 containing 1 M magnesium acetate. The assays were performed either in the presence or the absence of 25 mM methylthio adenosine diphosphate (ADPCP PL-biochemicals). The reaction was stopped by the addition of 0.2 ml 0.15 M ZnSO_4 and 0.2 ml 0.15 M $\text{Ba}(\text{OH})_2$. Following centrifugation ($2000 \times g$ for 10 min) activity in 0.9 ml supernatant was determined.

Results

Adenine and adenosine were rapidly incorporated into nucleotides in the fat cells. The time course and concentration dependence is shown in Fig. 1. At low concentrations the rate of incorporation was essentially linear for 30 min, while at higher concentrations (or 2 μM) there was a rapid initial rate of incorporation followed by a slower rate. The initial rate of incorporation was calculated in several experiments and their concentration dependence was plotted according to Lineweaver and Burk. Results from one typical experiment is shown in Fig. 2. The apparent K_m ranged between 1 and 5 μM for both substrates. Apparent V_{max} was 30–60 pmol/min/ 10^6 cells for adenine and 5–6 times less for adenosine (15 pmol/min/ 10^6 cells).

The radioactivity taken up by fat cells after labelling with adenine and before incubation was mainly in the form of nucleotides—of which ATP dominated (Fig. 3). During incubation

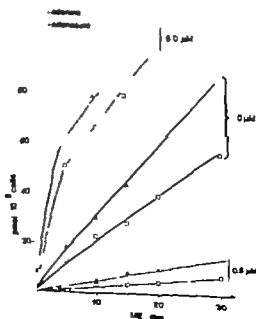


Fig. 1. Time-course of incorporation of labeled adenine and adenosine into nucleotides in fat cells. Results are duplicate determinations from a single experiment.

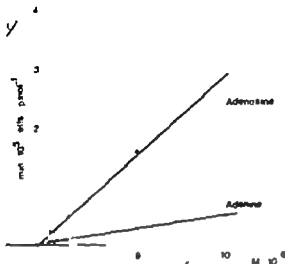


Fig. 2

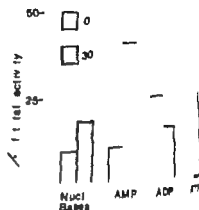


Fig. 3

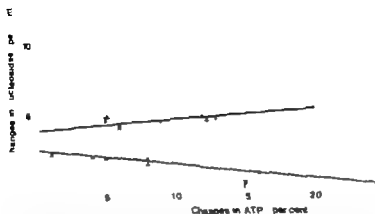
Fig. 2. Double reciprocal (Lineweaver-Burk) plot of the initial rate of adenine and adenosine incorporation into nucleotides.

Fig. 3. Distribution of radioactivity in fat cells before and after incubation for 30 min. Fat cells were prepared from 5 rats, incubated for 10 min with $10 \mu\text{Ci } ^{14}\text{C}$ -adenine, washed 6 times and incubated for 10 min in Krebs-Ringer phosphate buffer containing 0.1 mM dipyridamol. Radioactivity in the different fractions was determined by chromatography on long ($0.8 \times 15 \text{ cm}$) Dowex 1-2 (200-400 mesh) columns. The cells were incubated either in control buffer or in buffer containing $10 \mu\text{M}$ noradrenaline. There were no significant differences.

tion there was a progressive fall in ATP and a compensatory increase in mainly AMP in the nucleoside fraction. When the cells were incubated in the presence of dipyridamol (0.1 mM) there was a pronounced change in the distribution already after 30 min (Fig. 3). In the absence of dipyridamol the changes were qualitatively similar but less pronounced (not shown).

The formation of nucleosides (adenosine and inosine) is dependent upon dephosphorylation of AMP. The hydrolysis of AMP in fat cell homogenates was inhibited by approximately 70 per cent by AOPCP, suggesting that 5' nucleotidase is the major enzyme responsible for the hydrolysis in these cells. The apparent K_m for the reaction was $35 \mu\text{M}$ and the apparent V_{max} was $75 \text{ nmol min}^{-1} \text{ g}^{-1}$, essentially in agreement with the results of Newby *et al.* (1975). The rate of formation of nucleosides and bases is likely to increase with decreasing ATP levels for two reasons. Firstly, ATP is a potent inhibitor of the enzyme (cf. Newby *et al.* 1975). Secondly, the amount of substrate, AMP, is increased when ATP hydrolysis is increased. As shown in Fig. 4 such a relationship could be detected, even though it was of borderline significance, provided that there was no theophylline present in the incubation. The apparent inhibition of purine formation by theophylline may have at least two explanations.

In agreement with data obtained in brain and kidney (Tzusuiki and Newburgh-Fredholm-Hedqvist and Vernet 1978) we found that 1 mM theophylline inhibited



1 The relationship between changes in ^3H -ATP and in ^3H -nucleosides during 30 min incubation of fat cells. The distribution of radioactivity after labelling with ^3H -adenine was determined. At the beginning of incubation 48 ± 1 per cent of the radioactivity was in the form of ATP and 1 ± 1 per cent in the form of nucleosides. The cells were then incubated with or without different drugs in the presence of no phosphodiesterase inhibitor (Δ), in the presence of 0.1 mM papaverine (\circ) or in the presence of 1.0 mM theophylline. The relationship is calculated with conventional linear regression analysis. In the presence of no phosphodiesterase inhibitor or papaverine there was positive correlation ($r=0.352$, $p=0.05$), in the case of theophylline there was significant negative correlation ($r=-0.679$, $p<0.005$). The linear regression lines of y on x are indicated.

could be by 65 per cent in fat cells. Secondly theophylline may be diverting ATP to cyclic AMP channel labelled purines away from nucleoside formation. The data presented in Table I demonstrate the pronounced elevation of cyclic AMP levels in the presence of theophylline, but not papaverine, was combined with β -adrenoceptor stimulating agents.

The results in Table II demonstrate that noradrenaline and isoprenaline decreased H-ATP in cells labelled with ^3H -adenine. The effect of 3 μM noradrenaline was diminished, but not eliminated by 4 μM propranolol. This dose of propranolol similarly was not sufficient

2 Effect of noradrenaline (NA), isoprenaline (Iso) and methoxamine on adipocyte cyclic AMP levels. The cells were incubated for 5 min in the presence of theophylline (1.0 mM) or papaverine (0.1 mM) followed by 15 min in the presence of the drugs indicated. Mean \pm S.E. Number of observations in parentheses.

Drug	Cyclic AMP content pmol/ 10^6 cells	
	Theophylline	Papaverine
Drug	57 ± 0.3 (8)	34 ± 0.6 (8)
(3 μM)	128.1 ± 14.7 (4)	10.9 ± 1.2 (4)
propranolol (4 μM)	32.4 ± 1.7 (4)	3.2 ± 0.7 (4)
(3 μM)	134.1 ± 15.9 (4)	18.2 ± 0.4 (4)
propranolol (4 μM)	67.3 ± 11.1 (4)	3.2 ± 0.9 (4)
methoxamine (70 μM)	54 ± 0.8 (4)	2.1 ± 0.3 (4)

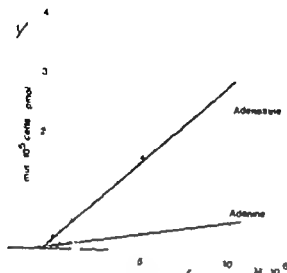


Fig. 2

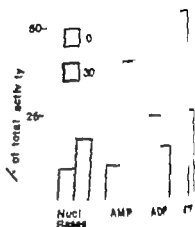


Fig. 3

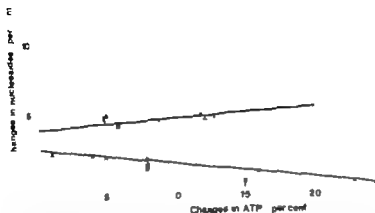
Fig. 2. Double reciprocal (Lineweaver-Burk) plot of the initial rate of adenine and adenosine incorporation into nucleotides.

Fig. 3. Distribution of radioactivity in fat cells before and after incubation for 30 min. Fat cells were prepared from 5 rats, incubated for 10 min with $10 \mu\text{Ci } ^3\text{H}$ -adenine washed 6 times and incubated for 10 min in Krebs Ringer phosphate buffer containing 0.1 mM dipyridamol. Radioactivity in the fractions was determined by chromatography on long ($0.8 \times 15 \text{ cm}$) Dowex 1×2 (200-400 mesh) column. The cells were incubated either in control buffer or in buffer containing $10 \mu\text{M}$ dipyridamol. There were no significant differences.

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The formation of nucleosides (adenosine and inosine) is dependent upon dephosphorylation of AMP. The hydrolysis of AMP in fat cell homogenates was inhibited by approximately 70 per cent by AOPCP suggesting that 5' nucleotidase is the major enzyme responsible for the hydrolysis in these cells. The apparent K_m for the reaction was $35 \mu\text{M}$ and the apparent V_{max} was $75 \text{ nmol min}^{-1} \text{ g}^{-1}$ essentially in agreement with the results of Newbury *et al.* (1975). The rate of formation of nucleosides and bases is likely to increase with decreasing ATP levels for two reasons. Firstly ATP is a potent inhibitor of the enzyme (cf. Newbury *et al.* 1975). Secondly the amount of substrate, AMP, is increased when ATP hydrolysis is increased. As shown in Fig. 4 such a relationship could be detected even though it was of borderline significance, provided that there was no theophylline present in the incubation medium. The apparent inhibition of purine formation by theophylline may have at least two explanations.

In agreement with data obtained in brain and kidney (Tsuruki and Newbury 1975; Fredholm, Hedqvist and Vernet 1978) we found that 1 mM theophylline inhibited 5'



The relationship between changes in H -ATP and in 3H -purines during 30 min incubation of fat. The distribution of radioactivity after labelling with H -adenine was determined. At the beginning incubation $48 \pm 1\%$ of the radioactivity was in the form of ATP and $1 \pm 1\%$ in the form of nucleosides. The cells were then incubated with or without different drugs in the presence of no phosphoramide inhibitor (Δ), in the presence of 0.1 mM papaverine (\circ) or in the presence of 1.0 mM theophylline. The relationship as calculated with conventional linear regression analysis. In the presence of no phosphoramide inhibitor or papaverine there was positive correlation ($r = 0.358$, $p = 0.05$), in the case of theophylline there was significant negative correlation ($r = -0.679$, $p = 0.005$). The linear regression lines of y on x are indicated.

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Table I. Effect of noradrenaline (NA), isoprenaline (iso) and methamphetamine on adipocyte cyclic AMP levels. The cells were incubated for 5 min in the presence of theophylline (1.0 mM) or papaverine (0.1 mM) followed by 15 min in the presence of the drugs indicated. Mean \pm S.E. Number of observations in parentheses.

Drug	Cyclic AMP content pmol/ 10^6 cells	
	Theophylline	Papaverine
drug		
($3 \mu M$)	5.7 ± 0.3 (8)	3.4 ± 0.6 (8)
propranolol ($4 \mu M$)	128.1 ± 14.7 (4)	10.9 ± 1.2 (4)
($3 \mu M$)	12.4 ± 1.7 (4)	3.2 ± 0.7 (4)
propranolol ($4 \mu M$)	134.1 ± 15.5 (4)	10.2 ± 0.4 (4)
propranolol ($4 \mu M$)	67.3 ± 11.1 (4)	3.2 ± 0.9 (4)
flunarizine ($70 \mu M$)	3.4 ± 0.8 (4)	2.1 ± 0.3 (4)

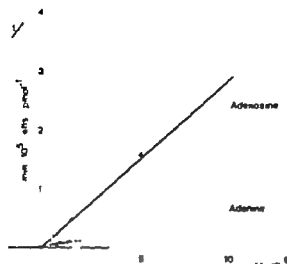


Fig. 2

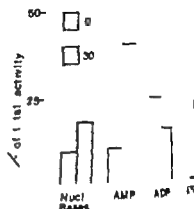


Fig. 3

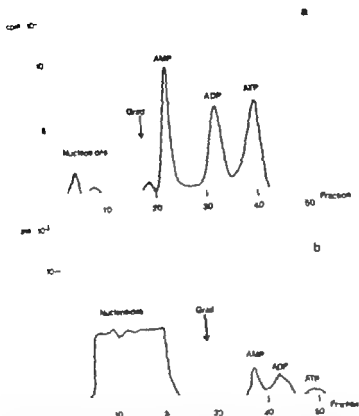
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The formation of nucleosides (adenosine and inosine) is dependent upon dephosphorylation of AMP. The hydrolysis of AMP in fat cell homogenates was inhibited by approximately 70 per cent by AOPCP suggesting that 5' nucleotidase is the major enzyme responsible for the hydrolysis in these cells. The apparent K_m for the reaction was $35 \mu\text{M}$ and the apparent V_{max} was $75 \text{ nmol min}^{-1} \text{ g}^{-1}$ essentially in agreement with the results of Newby *et al.* (1975). The rate of formation of nucleosides and bases is likely to increase with decreasing ATP levels for two reasons. Firstly ATP is a potent inhibitor of the enzyme (cf. Newby *et al.* 1975). Secondly the amount of substrate, AMP, is increased when ATP hydrolysis is increased. As shown in Fig. 4 such a relationship could be detected even though it was of borderline significance, provided that there was no theophylline present in the incubation medium. The apparent inhibition of purine formation by theophylline may have at least two explanations.

In agreement with data obtained in brain and kidney (Tsuruki and Newburgh 1978; Fredholm, Hedqvist and Vernet 1978) we found that 1 mM theophylline inhibited



6 A. Distribution of radioactivity in fat cells prelabelled with ^3H -adenine. The separation was performed on long Dowex 1 column eluted with hydrochloric acid gradient.

6 B. Distribution of radioactivity in the perfusion effluent from the same cells as in Fig. 6 A. Note marked shift towards nucleosides in the effluent, as compared to the distribution in the cells.

7) and noradrenaline had no significant further effect (to $11.8 \pm 2.6\%$, $n=5$). When the cells were incubated with theophylline the nucleoside release was smaller in magnitude (see 4) and there were no consistent effects of catecholamines.

The fall in ATP commonly seen following incubation with lipolytic drugs has been linked to accumulation of lipolytic products, primarily fatty acids (Angel *et al* 1971). In order to study a possible direct effect of noradrenaline on purine release from fat cells we therefore used the perfused fat cell system (Allen *et al* 1973, Hjermadal and Sollevi 1978). This system has the advantage that lipolytic products do not accumulate since the medium is constantly changed.

After the initial equilibration period of 10–15 min there was a fairly steady outflow of radioactivity from ^3H -adenine prelabelled perfused fat cells (Fig. 5). This basal outflow of radioactivity amounted to 0.49 ± 0.04 of the cellular content per min ($n=11$). As can be seen in Fig. 5 most of the radioactivity released was nucleoside material, of which adenosine

TABLE II Distribution of radioactivity as ATP and as nucleosides and bases after incubation with; Incubation for 45 min in the presence of papaverine + indicated drug. Mean of 44 determinations. Results are expressed as per cent of total radioactivity

	ATP	Nucleosides & Bases
Control	40.5 \pm 0.6	3.9 \pm 0.6
NA 3 μ M	27.5 \pm 0.5*	6.3 \pm 0.3
NA + propranolol 4 μ M	34.0 \pm 2.0	4.6 \pm 0.1
Iso 0.3 μ M	33.0 \pm 6.0	5.8 \pm 1.0
Iso + propranolol 4 μ M	31.5 \pm 1.5	4.6 \pm 0.3
Methoxyamine 70 μ M	38.0 \pm 1.0	5.8 \pm 0.6

p < 0.01 vs. control

■ 0.01 \pm no propranolol

to completely block the action of noradrenaline on cyclic AMP (Table I). Noradrenaline also increased the fraction of radioactivity recovered in the form of nucleosides and significantly when the cells were incubated with papaverine (Table II). This effect of adrenaline was antagonized by propranolol. When the cells were incubated in the presence of dipyrindamol (0.1 mM) the accumulation of purine nucleosides was higher (102 %)

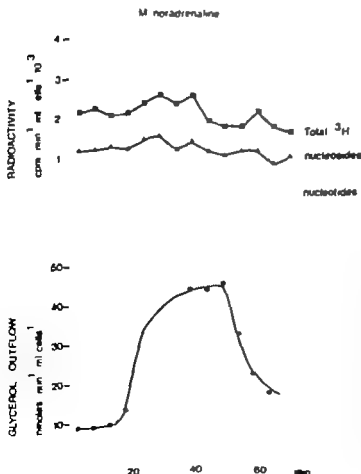


Fig. 5. Lipolysis and outflow of radioactivity from ^3H -adrenaline labelled perfused fat cells stimulated by 1 μM noradrenaline. Radioactivity was separated on nucleoside and nucleotide fractions on small Dowex 1 column. Glycerol as measured as described by Hjendahl (1971). Note the very minor outflow of nucleoside outflow during stimulation with dose of noradrenaline causing substantial lipolysis response.

argument that it acts—at least partly—by interfering with endogenous adenosine (Angel *et al.* 1972, Ebert and Schwabe 1973). Accumulated adenosine in the incubation medium, and under some circumstances accumulation was enhanced by noradrenaline. Moreover the effect of noradrenaline was antagonized by propranolol. However the purine formation may not necessarily be linked to lipolysis. During these conditions there was a marked reduction of fat cell ATP levels, in agreement with the results of others (Hepp *et al.* 1968, Bühler and Jeanrenaud 1971, Angel *et al.* 1971). It has been shown that such a depression of ATP levels may be caused by lipolysis in so far as it appears to be caused by accumulation of fatty acids (Angel *et al.* 1971). The present finding that there was very little increase in purine release by noradrenaline in perfused fat cells where accumulating fatty acids are continuously washed away may be taken as an argument that the noradrenaline-induced nucleoside accumulation in incubated fat cells is not directly caused by lipolysis. Furthermore, when the cells were incubated in the presence of dipyridamol or theophylline no consistent effects of cyclic catecholamines on purine nucleoside accumulation were found. Similarly Schwabe *et al.* (1973) were unable to detect a significant effect of noradrenaline on adenosine levels in cell incubates.

When lipolytic stimuli increase accumulation of purine nucleosides, including adenosine, they thus fit due to the depression of ATP levels that can be caused by accumulating lipolytic products such as fatty acids, rather than to a direct effect of the lipolytic stimuli. Cell ATP levels may also be depressed by decreased oxygen supply. The finding that perfusion, in contrast to mere aeration, of the perfusion buffers led to a decreased rate of purine nucleoside release suggests that the oxygen supply is also of importance.

The intact adipose tissue adenosine-like material was released by sympathetic nerve stimulation (Fredholm 1976). This release was prevented by α -adrenoceptor blockade. The relationship between decrease in ATP levels and enhanced purine nucleoside release has also been demonstrated under *in vivo* conditions (Fredholm *et al.* 1977). It is interesting to note that during sympathetic nerve stimulation, which causes vasoconstriction concomitantly with lipolysis, there is a decrease in tissue pO_2 (Fredholm *et al.* 1976) as well as an increase in the tissue content of FFA (Fredholm 1970). Sympathetic nerves provide the most important stimulus for lipolysis *in vivo*. Therefore, ATP levels may decrease during activation of lipolysis which, in turn, will lead to increased formation of adenosine.

The present findings thus support the idea that adenosine is not a feed back regulator of lipolysis in the strict sense of the word. The formation of adenosine is not causally linked to lipolysis, but does appear to be linked to changes in cellular ATP levels. However, remarkable changes in ATP are caused by the same stimuli that cause lipolysis. A role for adenosine as a physiological modulator of lipolysis thus remains a distinct possibility.

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was a major component (thin layer chromatography not shown). Fig. 6 a shows the distribution of the radioactivity in an aliquot of prelabelled cells and Fig. 6 b the distribution of radioactivity in the perfusion effluent from the same cells. It can be seen that most of the radioactivity in the cells is present in the nucleotide stores, whereas most of the radioactivity in the effluent is nucleoside material.

Stimulation of lipolysis in the perfused fat cells by 1 μ M noradrenaline caused at minor if any increase in nucleoside outflow as shown in Fig. 5. On the average 1 μ M adrenaline increased outflow of radioactivity from 3 H-adenine prelabelled fat cells by 12 ± 6 (n=8 N.S.). Similarly 1 μ M isoprenaline had an insignificant effect on outflow (increase by $14 \pm 13\%$, n=4). The outflow of nucleosides was, however, influenced by oxygenation of the perfusion buffer. Thus, the fractional release of radioactivity was reduced by $34 \pm 7\%$ (n=5 $p < 0.01$) in cells perfused with oxygenated buffer as compared to identical cells perfused with aerated buffer. The lipolytic response of the cells was consistently altered by this change in PO_2 .

Discussion

The present study confirms and extends earlier reports of rapid uptake and phosphorylation of adenine and adenosine (Kuo and De Renzo 1969, Ebert and Schwabe 1973). The rate of incorporation into nucleotides was faster with adenine as substrate and consequently adenine was used for subsequent studies of the formation of purines from prelabelled nucleoside stores.

Already during basal conditions (*i.e.* in the absence of lipolytic drugs) there was a appreciable formation of purine nucleosides. In the perfused fat cell system, where the nucleosides are continuously removed, the rate of formation amounted to about 0.5% of the total cellular radioactive stores per min. Assuming homogenous labelling of the nucleotide stores and an ATP content of 1-2 nmol/ 10^4 cells (Schwabe *et al.* 1974, Fredholm 1977) this would correspond to the release of 5-10 pmol adenosine/min/ 10^4 cells. Interestingly this figure agrees well with the initial rate of accumulation of endogenous adenosine during the incubation of fat cells (Schwabe *et al.* 1973).

The accumulation of purines was markedly inhibited by theophylline. Thus, theophylline interferes with adenosine mechanisms in fat cells in several different ways. (1) The lipolytic and cyclic AMP depressing effect of adenosine is competitively inhibited by theophylline (Fain 1973, Ebert and Schwabe 1973, Fredholm 1978), (2) the formation of adenosine is inhibited by theophylline at least partly due to inhibition of 5'-nucleotidase (Fain and Newburgh 1974, Fredholm *et al.* 1978), and (3) theophylline may inhibit adenosine uptake by fat cells (Fain *et al.* 1978). Although the latter effect is minor (Schwabe 1973), it may reflect hindrance not only of inward but also outward transport of adenosine over the fat cell membrane. These findings further underscore the hazards of assuming that theophylline acts exclusively by virtue of its (poor) phosphodiesterase inhibitory action. The finding that theophylline is far more potent as a stimulator of cyclic AMP accumulation in fat cells than the considerably more potent phosphodiesterase inhibitors papaverine and dipyridamol (Schwabe *et al.* 1972, Hjermidahl and Fredholm 1976) may instead

- MAN, H. and R. B. FRIEDMAN, Cyclic AMP-dependent and independent inhibition of lipolysis by norepinephrine and decreased pH. *Acta physiol. scand.* 1976, 96, 170-179.
- MAN, P. and A. SOLLEVI, Antilipolytic effect of adenosine in isolated perfused fat cells. *Acta physiol. scand.* 1978, 103, 270-274.
- MAN, P., R. B. FRIEDMAN and A. SOLLEVI, Release of adenosine-like material from isolated "perid" fat cells. *Acta physiol. scand.* 1978, 102, 81A.
- J. F. and E. C. DE RINZO, A comparison of the effects of lipolytic and antilipolytic agents on adenosine 3,5-monophosphate levels in adipose cells as determined by prior labeling with adenosine-8-¹⁴C. *Mol. Chem.* 1969, 244, 2252-2260.
- J. A. C. J. P. LUYD and C. N. HALL, The properties and extracellular location of 5'-nucleotidase in the fat-cell membrane. *Biochem. J.* 1975, 146, 625-633.
- I. and H. MCI WANE, Metabolism of ¹⁴C-adenosine and derivatives by cerebral tissues, superfused and structurally stimulated. *Biochem. J.* 1972, 126, 965-973.
- ELL, M., Metabolism of isolated fat cells. I. Effect of hormones on glucose metabolism and lipolysis. *Mol. Chem.* 1964, 239, 373-380.
- TY, W. J. B. M. SCHWOT and J. J. H. H. M. DE PONT, Adenosine 3,5-monophosphate phosphorylation in liver homogenates. *Biochem. Biophys. Acta* 1973, 313, 376-381.
- AM, U. and R. EMERY, Stimulation of cyclic adenosine 3,5-monophosphate accumulation and release in fat cells by adenosine deaminase. *Neuro-Schwarzenberg Arch. Pharmacol.* 1974, 232, 33-44.
- AM, U. S. BERCHOT and R. EMERY, Activation and inhibition of lipolysis in isolated fat cells by various agonists of cyclic AMP phosphorylation. *Neuro-Schwarzenberg Arch. Pharmacol.* 1972, 273, 62-74.
- AM, U., R. EMERY and H. C. EASLER, Adenosine release from isolated fat cells and the significance of the effects of hormones on cyclic 3,5-AMP levels and lipolysis. *Neuro-Schwarzenberg Arch. Pharmacol.* 1973, 276, 133-148.
- AM, U., R. EMERY and H. C. EASLER, Adenosine release from fat cells: effect on cyclic AMP levels and hormone actions. *Adv. cyclic Nucleic Res.* 1975, 3, 549-564.
- WAM, U. P. S. SCHWOT and R. EMERY, Facilitation by adenosine of the action of insulin on the phosphorylation of adenosine 3,5-monophosphate, lipolysis, and glucose oxidation in isolated fat cells. *or J. Biochem.* 1974, 46, 537-543.
- ELL, J. and R. W. NEWBORN, Inhibition of 5'-nucleotidase in rat brain by methylxanthines. *J. Neurochem.* 1975, 25, 895-896.
- JY, B. P. W. C. DUCKWORTH and S. S. SOLOMON, Perfusion of isolated rat adipose cells. Modulation of lipolysis by adenosine. *J. clin. Invest.* 1977, 60, 442-448.

References

- ALLEN D D, E. E. LARGIS, E. A. MILLER and J. ASHMORE. Continuous monitoring of lipolysis in perfused isolated fat cells. *J. appl. Physiol.* 1973. 34. 125-127.
- AMIEL, A. L. S. DESAI and M. L. HALPERIN. Reduction in adipocyte ATP by lipolytic agents alters intracellular free fatty acid accumulation. *J. Lipid Res.* 1971. 12. 203-213.
- BAHLER, I. and B. JEANRENAUD. ATP content of isolated fat cells. Effects of insulin, ouabain, and lipolytic agents. *Biochim. biophys. Acta* (Amst.) 1970. 202. 496-506.
- BÖHME, E. and G. SCHULTZ. Separation of cyclic nucleotides by thin-layer chromatography on ethylenimine cellulose. In *Methods of Enzymology* vol. 38 (Eds. J. G. Hardman and B. W. O. O'Keefe). Academic Press, New York, 1974. 27-38.
- BROWN, B. L., R. P. EAKINS and J. D. M. ALBANO. Saturable binding assay for cyclic AMP using calyculin A binding protein. *Adv. Cyclic Nucleic Res.* 1972. 2. 25-40.
- EBERT, R. and U. SCHWARZ. Studies on the antilipolytic effect of adenosine and related compounds in isolated fat cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 1973. 278. 247-259.
- FAIM, J. N. Inhibition of adenosine cyclic 3',5'-monophosphate accumulation in fat cells by N^6 -(phenylisopropyl)adenosine and related compounds. *Molec. Pharmacol.* 1973. 9. 595-604.
- FAIM, J. N., R. H. PORTER and W. F. WARD. Effects of adenosine nucleosides on adenylyl cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation and lipolysis in fat cells. *J. Biol. Chem.* 1972. 247. 6866-6872.
- FAIM, J. N. and R. E. SHEPHERD. Hormonal regulation of lipolysis: role of cyclic nucleotides and free fatty acids. Proc. Midwest regional conference on endocrinology and metabolism. R. P. Press, New York, 1976.
- FAIM, J. N., R. E. SHEPHERD, C. C. MALBON and F. J. MORENO. Hormonal regulation of the breakdown of triglyceride in adipocytes. In *The physiology of lipids and lipoproteins in health and in disease* (Ed. L. Dietschy). American Physiological Society, Bethesda, 1978. In press.
- FAIM, J. N. and P. B. WISER. Effect of adenosine deaminase on cyclic adenosine monophosphate accumulation, lipolysis and glucose metabolism of fat cells. *J. Biol. Chem.* 1975. 250. 1027-1034.
- FREDHOLM, B. B. Studies on the sympathetic regulation of circulation and metabolism in isolated or subcutaneous adipose tissue. *Acta physiol. scand.* 1970. 80. Suppl. 354. 1-47.
- FREDHOLM, B. B. Release of adenosine-like material from isolated perfused dog adipose tissue by sympathetic nerve stimulation and its inhibition by adrenergic α -receptor blockade. *Acta physiol. scand.* 1976. 96. 422-430.
- FREDHOLM, B. B. Effect of adenosine, adenosine analogues and drugs inhibiting adenosine uptake on lipolysis in rat fat cells. *Acta physiol. scand.* 1978. 102. 191-198.
- FREDHOLM, B. B. and P. HUENDAHL. Inhibition by adenosine of adenosine 3',5'-cyclic monophosphate accumulation and lipolysis in isolated rat fat cells. *Acta physiol. scand.* 1976. 96. 160-169.
- FREDHOLM, B. B. and A. SOLLEVI. Antilipolytic effect of adenosine in dog adipose tissue in vivo. *Acta physiol. scand.* 1977. 99. 254-257.
- FREDHOLM, B. B. and A. SOLLEVI. Control of lipolysis and circulation in adipose tissue by endogenous adenosine. *Acta physiol. scand.* 1978. 102. 77A-78A.
- FREDHOLM, B. B., E. BELFRAH and E. BLANCHER. Changes in ATP and cyclic nucleotide levels during sympathetic nerve stimulation in canine subcutaneous adipose tissue in vivo. *Acta physiol. scand.* 1977. 99. 313-322.
- FREDHOLM, B. B., P. HEDQVIST and L. VERNET. Effect of theophylline and other drugs on rabbit cyclic nucleotide phosphodiesterase 5-nucleotidase and adenosine deaminase. *Biochem. Pharmacol.* 1978. In press.
- FREDHOLM, B. B., B. LINDE, R. L. PREWITT and P. C. JOHNSON. Oxygen uptake and tissue oxygenation during adrenergic stimulation of canine subcutaneous adipose tissue. *Acta physiol. scand.* 1978. 102. 48-59.
- GENTRY, M. K. and R. A. OLSSON. A simple specific radiolabelled assay for 5-nucleotidase. *Acta Biochem.* 1975. 64. 624-627.
- HART, D. D. R. CHALLONER and R. H. WILLIAMS. Studies on the action of insulin in isolated adipose cells. I. Stimulation of incorporation of ^{32}P labelled inorganic phosphate into mononucleotides in the absence of glucose. *J. Biol. Chem.* 1968. 243. 4020-4026.
- HUENDAHL, P. Studies on the antilipolytic effect of adenosine. *Acta physiol. scand.* 1976. 97. Suppl. 434. 1-10.

Fords, the Spitzbergen reindeer is much fatter in the fall than their Norwegian relatives. This leads to the question of whether such marked fattening capability is reflected in a seasonal pattern different from that of other reindeer.

Thus, the purpose of the present study was to determine the levels of growth hormone (GH) in free ranging Spitzbergen reindeer both during late winter when food is in supply and during late summer before the rut, when their nutritional status is optimal. In addition, the concentration of cortisol was to be measured as a tentative indicator of

Material and methods

of 33 Spitzbergen reindeer (*Rangifer tarandus pliarcticus*) are used. Blood samples were collected on two expeditions to Svalbard in 1976. During the first expedition (Adventdalen, March 24–April 8, 1976) winter blood samples were collected from 30 adult animals (20 females and 10 males) and from 5 calves accompanying their mothers. The samples were taken by jugular venopuncture using evacuated sampling tubes, immediately after capture of the animals. During the second expedition (Groefjordene, June 13–27, 1976) late-summer blood samples were collected as described above from 14 adult animals (8 females and 6 males) and from 5 calves accompanying their mothers. The serum fractions were frozen and stored at -18°C till analysis.

Growth hormone (GH) was measured by the radioimmunoassay of Wool and Selenkow (1968) as modified by Klotz and Klotz (1973). Bovine growth hormone (a gift from NIH-Bethesda, Batch no. B1003A) was used as standard, and rabbit anti-BGH as anti-serum.

Cortisol (C) was measured according to the method of Webb (1972) as modified by Foss (1973). Intra-assay coefficient of variation was determined by Corupack® Assay Kits (Amersham, England). Between-assay values were tested statistically by use of Student's *t*-test. Results are presented as Mean \pm S.E.

Results

No difference between sexes was found for any of the hormones tested. The results from males and females are therefore pooled.

Fig. 1 the summer-winter variation in growth hormone for the Spitzbergen and Norwegian reindeer is shown. A conspicuous ($P < 0.01$) increase in the level of growth hormone is evident in winter both in calves and in adult Spitzbergen reindeer (10.8 ± 1.4 ng/ml ($n = 5$) vs 71.5 ± 19 ng/ml ($n = 4$) for calves, and 11.2 ± 0.5 ng/ml ($n = 14$) and 73.3 ± 11 ng/ml ($n = 30$) for adults). Winter levels of growth hormone were higher in Spitzbergen reindeer than in Norwegian reindeer (21.6 ± 1.6 ng/ml (calves, $n = 14$) and 20.3 ± 0.9 ng/ml (adults, $n = 9$) in the latter while summer levels were higher in the Norwegian reindeer (31.9 ± 2.2 ng/ml for calves ($n = 12$) and 25.5 ± 1.5 ng/ml for adults ($n = 24$)). Seasonal changes in growth hormone in Norwegian reindeer were small, but summer levels tended ($P < 0.05$) to be somewhat higher than winter levels.

Seasonal change in cortisol levels were recorded for the adult groups. 5.7 ± 0.6 $\mu\text{g}/100$ ml ($n = 14$) and 4.7 ± 0.5 $\mu\text{g}/100$ ml ($n = 21$) for Spitzbergen and Norwegian reindeer respectively in summer and 5.8 ± 0.3 $\mu\text{g}/100$ ml ($n = 30$) and 5.1 ± 0.8 $\mu\text{g}/100$ ml ($n = 15$) for the expanding groups in winter. The Norwegian calf group was generally lower in cortisol than the other groups. 2.8 ± 0.3 $\mu\text{g}/100$ ml ($n = 12$) ($P < 0.01$) during summer and 3.5 ± 0.5 $\mu\text{g}/100$ ml ($n = 13$) ($P < 0.05$) during winter while the Spitzbergen calf group did not differ significantly from the adult groups (5.0 ± 1.7 $\mu\text{g}/100$ ml ($n = 5$) (summer), and 6.7 ± 0.7 $\mu\text{g}/100$ ml ($n = 4$) (winter) (Fig. 2).

The Spitzbergen reindeer—a winter-dormant ungulate?

By

TATA RINGBERG

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Abstract

RINGBERG T. *The Spitzbergen reindeer—a winter-dormant ungulate?* Acta physiol. sc. 1979 105: 268-273

Seasonal changes in serum levels of growth hormone, cortisol and thyroxine in calves and adult Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) were measured and compared to those previously found in Norwegian reindeer (*R. tarandus*). Cortisol did not differ significantly between seasons and was similar between the subspecies. Growth hormone and thyroxine exhibited highly significant seasonal changes. Subspecific differences: winter levels of growth hormone were much higher than summer levels in Spitzbergen reindeer while Norwegian reindeer differed very little from season to season. Both Spitzbergen and Norwegian reindeer had markedly reduced thyroxine levels during winter but the values in the former were much lower than those from the latter. In summer however the levels are equal. The high levels of growth hormone and low levels of thyroxine in the Spitzbergen reindeer during winter are indicative of high lipolytic activity and reduced metabolic rate respectively.

The Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) occupy one of the northernmost habitats of all cervidae, and have been isolated on the archipelago of Svalbard (Spitzbergen) for an unknown number of centuries. The climate of Svalbard is influenced by a west-going branch of the Gulf Stream and the temperatures therefore do not reach the extremes of more typical high-arctic areas. Strong winds of high moisture and occasional periods of above-freezing temperatures in late winter however result in icing of the snow, thus covering the range and reduce the amount of vegetation accessible to the reindeer (Ringberg 1977). Also the search for food may be impaired by a winter night of almost complete darkness for nearly 3 months. Consequently the food availability is limited and the reindeer must heavily rely upon the fat reserves gained and stored during summer for their winter survival.

Studies on the body composition of the Spitzbergen reindeer in late summer (Ringberg *et al.* 1977) have shown the fat/muscle ratio to be much higher than that of Norwegian reindeer (Ringberg, unpublished) although the total body weight is about the same.

Mean temp. for the warmest month, July is +6°C, and for the coldest month, December is -16°C. Data from the Stat. Meteorological Institut of Norway.

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As the purpose of the present study was to determine the levels of growth hormone (GH) in free ranging Spitzbergen reindeer both during late winter when food is in supply and during late summer before the rut, when their nutritional status is optimal. In addition, the concentration of cortisol was to be measured as a tentative indicator of stress.

Material and methods

Of 53 Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) were used. Blood samples were collected on two expeditions to Svalbard in 1976. During the first expedition (Advestfjella, March 24–April 8), 10 ml blood samples were collected from 30 adult animals (20 females and 10 males) and from 10 calves accompanying their mothers. The samples were taken by jugular venopuncture using evacuated mg tubes, immediately after capture of the animals. During the second expedition (Grønfjorden, 13–27 June 1976) late-summer blood samples were collected as described above from 14 adult animals (8 males and 6 females) and from 5 calves accompanying their mothers. The serum fractions were frozen and stored at -18°C in bottles.

Growth hormone (GH) was measured by the radioimmunoassay of Wool and Schenkow (1968) as modified by Foss and Bloom (1973). Bovine growth hormone (a gift from NIH-Bethesda, Batch no. B1003A) was used as standard, and rabbit anti-BGH as anti-serum.

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The Spitzbergen reindeer (*Rangifer tarandus platyrhynchus*) occupy one of the northernmost habitats of all cervidae, and have been isolated on the archipelago of Svalbard (Spitzbergen) for an unknown number of centuries. The climate of Svalbard is influenced by a cold going branch of the Gulf Stream and the temperatures therefore do not reach the evenness of more typical high-arctic areas. Strong winds of high moisture and occasional periods of above freezing temperatures in late winter however result in melting of the snow which covers the range and reduce the amount of vegetation accessible to the reindeer (Ringberg 1977). Also the search for food may be impaired by a winter-night of almost complete darkness for nearly 3 months. Consequently the food availability is limited and the reindeer must heavily rely upon the fat reserves gained and stored during summer for their winter survival.

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Material and methods

of 53 Spitzbergen reindeer (*Rangifer arcticus platyacris*) were used. Blood samples were collected on two expeditions to Svalbard in 1974. During the first expedition (Adventdalen, March 24–April 8, 1974) blood samples were collected from 30 adult animals (20 females and 10 males) and from 10 calves accompanying their mothers. The samples are taken by jugular venipuncture using evacuated glass tubes, immediately after capture of the animals. During the second expedition (Grønfjorden, 13–27/1974) late-summer blood samples are collected as described above from 14 adult animals (10 females and 4 males) and from 5 calves accompanying their mothers. The serum fractions are frozen and stored at -18°C till analysis.

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Cortisol (T_1) was measured according to the method of Webb (1972) as modified by Foss (1973). All in-vitro and in-vivo samples were determined by Cortapeck Assay Kits (Amersham, England). Differences between mean values were tested statistically by use of Student's *t*-test.

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Fig. 3 it is shown that the thyroxine level in the two calf groups are identical during summer 261 ± 17 nmol/l ($n = 5$) for Spitzbergen, and 249 ± 10 nmol/l ($n = 12$) for Norwegian and likewise, that the adult level of thyroxine during summer is similar 216 ± 15 nmol/l ($n = 14$) for Spitzbergen and 223 ± 7 nmol/l ($n = 24$) for Norwegian reindeer. In the thyroxine levels of both subspecies are markedly reduced, but the decrease in Spitzbergen reindeer is considerably larger than that in Norwegian reindeer 69 ± 7 nmol/l ($n = 4$) vs. 144 ± 9 nmol/l ($n = 13$) for Spitzbergen and Norwegian calves, respectively in winter and 80 ± 5 nmol/l ($n = 30$) vs. 157 ± 5 nmol/l ($n = 16$) for adults, respectively. The winter thyroxine concentration in the Spitzbergen reindeer is only about one of the summer concentration and 50% lower than the winter levels of Norwegian reindeer. The seasonal changes are statistically highly significant $P < 0.001$ for Spitzbergen reindeer and $P = 0.01$ for Norwegian reindeer.

Discussion

Growth hormone plays a key role in maintaining blood homeostasis of fatty acids and glucose by stimulating the mobilization of free fatty acids from triglycerides in depot fat and by stimulating the mobilization of glucose from the liver and by exerting a marked anabolic effect (Olick *et al.* 1965, Talwar *et al.* 1975). The high growth hormone concentrations in winter blood samples from Spitzbergen reindeer are therefore strongly suggestive of an energy metabolism which is dependent upon the mobilization of stored fat to a higher degree than that for Norwegian reindeer.

However, during periods of stress such as pain, starvation or exposure to cold, the level of growth hormone is often elevated as part of the physiological response to stress (Roth 1963, Feldman and Brown 1967, Olick and Trenkle 1973). Thus, the high growth hormone levels could also indicate, that the winter conditions on Svalbard with scarce food, darkness and high windchill are tough on the animals, rather than being reflections of energy metabolism.

When an animal is exposed to a variety of noxious, or potentially noxious stimuli, there is an increase in the secretion of adrenocorticotrophic hormone (ACTH) and, consequently, an increase in circulating cortisol or corticosterone (Noble 1950, 1955). Thus, if stress in reindeer is reflected in elevated cortisol levels like it is in sheep (Pinaretto and Vickery 1972), cattle (Mason and Buckland 1976) and moose (Frantzman *et al.* 1975), the lack of a significant increase in circulating cortisol in Spitzbergen reindeer during winter may indicate that these animals are not under severe thermal or nutritional stress. The profound seasonal changes in growth hormone levels in Spitzbergen reindeer therefore most likely reflect a specific metabolic adaptation which includes a remarkable fat-storing capacity in periods with low food supply and a well-developed ability for retrieval of stored body fat during periods of food scarcity.

The most well known effect of thyroxine is acceleration of metabolic rate measured as increased oxygen consumption and heat production (Saher 1950), but thyroxine also influences behavior. Hyperthyroidism results in nervousness and hyperactivity while hypothyroidism is associated with lethargic behavior (Rawson *et al.* 1955). Reduced feed intake

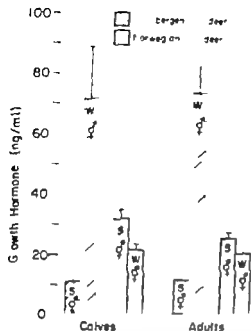


Fig. 1

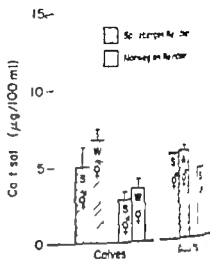


Fig. 2

Fig. 1. Growth hormone levels in serum from Spitzbergen reindeer collected in August (S) and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg, Larsen *et al.* 1978 a). Each value is given as Mean \pm S.E. (Number of animals in brackets)

Fig. 2. Cortisol levels in serum from Spitzbergen reindeer collected in August (S) and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg, Larsen *et al.* 1978 b). Each value is given as Mean \pm S.E. (Number of animals in brackets)

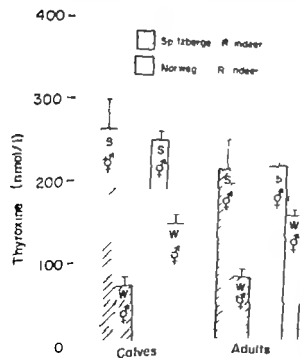


Fig. 3. Thyroxine (T) levels in serum from Spitzbergen reindeer collected in August (S) and in March (W). Data are compared to similar results from Norwegian semidomesticated reindeer (Ringberg, Larsen *et al.* 1978 a). Each value is given as Mean \pm S.E. (Number of animals in brackets)

1. E., Population dynamics in two subpopulations of reindeer in Svalbard. *Arctic and Alpine Research* 9: 369-381.
2. E., T. RICHARD LUND-LARSEN and R. SÆVINGGAARD, Kjusj, Isak/Skoddajon/Isakvæmdyngstakk. MAR | NORGE. Svalbard-projektet. Rapport No. 4. 1977 100-104.
3. LUND-LARSEN, T. J. KORTAD and A. AARVANG, Seasonal changes in serum levels of aldosterone, sodium and morphine loss in the reindeer (*Rangifer tarandus*). *Comp. Biochem. Physiol.* 1978. 60A, 386.
4. LUND-LARSEN, T. E. JACOBSEN, M. RYD and J. KROG, Seasonal changes in levels of growth hormone, somatomedin and thyroxine in free ranging, semi-domesticated Norwegian reindeer (*Rangifer tarandus*, L.) *Comp. Biochem. Physiol.* 1978. 60A, 123-126.
5. J. M. GLICK, R. S. YALOW and E. A. BERSON, Hypophysectomy. A potent stimulator to secretion of thyroid hormones. *Science* 1963. 140 947-948.
6. W. T. The chemistry and physiology of the thyroid hormones. In *The Hormones*. Eds. B. FROST and R. V. THOMAS, Vol. II, Academic Press, New York. 1959. 181-299.
7. J. S. L. J. VERME, J. J. OZOGA and A. W. EMMERSON, Nutritional effects on thyroid activity and blood glucose of introduced deer. *J. Wildl. Mgmt.* 1972. 36. 1041-1052.
8. G. P. M. R. PANDAY, N. KUMAR, S. N. S. HANJIAN, R. K. SAXENA, R. KRISHNARAJ and S. L. TAL, Mechanism of action of pituitary growth hormone. *Recent Progr. Hormone Res.* 1975. 31 141.
9. A. M. Assay of serum thyroxine. Radioassisted determination of thyroxine (T_4) iodine. *Stand. Arch. Clin. Chem.* 1972. 7 261-266.
10. Energy metabolism in the Roe deer (*Capreolus capreolus*). *Acta Theriologica* 1977. 22. 3-24.
11. M. S. and H. A. SILENTOW, Charcoal-Dextran radioimmunoassay of human growth hormone. *reiner (Kb)* 1968. 57 109-114.

has been shown to reduce the plasma level of thyroxine (T₄) in white-tailed deer (Se 1972) and in goats (Abdullah and Falconer 1977), and to reduce resting metabolic rate in deer (Weiner 1977). In both Norwegian and Spitzbergen reindeer thyroxine is lower in winter than in summer but in the latter the difference is very large, in fact, from the low winter concentrations of thyroxine in Spitzbergen reindeer one would noticeable behavioral changes.

It is well known that Spitzbergen reindeer exhibit a very "tame" behavior while human interference during summer elicits a "normal" flight reaction. Based on data presented in this report I wish to put forward the hypothesis that the winter dormancy is caused by the very low thyroxine levels, and is presumably associated with metabolic rates. In the Svalbard-environment devoid of reindeer predators, the "winter dormancy" could be possible as a mode of energy conservation for well insulated animals. The adjustments of lipid metabolism, the lack of stress symptoms, the apparent winter dormancy all suggest that the Spitzbergen reindeer are uniquely adapted to their high arctic island habitat.

I am indebted to Professor O. P. Foss, Oslo City Hospital, Ullevål, for performing the thyroxine analyses. The skilful technical assistance of chem. ing. V. Schueller-Jacobsen, Dept. of Physiology, University of Norway is also gratefully acknowledged. Professor J. Krog, Institute of Zoophysiology, University of Oslo, Norway introduced me to the field of Arctic Biology and supported all stages of the project. Logistic assistance on Svalbard was supplied by Professor J. Krog and by the Norwegian Maritime Administration of N. A. Ørtengren. This project was supported by the Norwegian Research Council for Science and the Humanities as Project No. D 68 70-5, Grant No. GM 10402, NIH, U.S. Public Health Service and ERDA Contract EY 76-5-06-2229 TA No. 3.

References

- ABDULLAH, R. and J. R. FALCONER, Responses of thyroid activity to feed restriction in the goat. *Biol. Sci.* 1977 30: 207-216.
- FELDMAN, J. and G. M. BROWN, Endocrine responses to electric shock and a cold stress in the rhesus monkey: cortisol and growth hormone. *Psychoneuroendocrinology* 1967 1: 231-241.
- Foss, O. P. Chemical determination of thyroxine-sodium in serum by a radiochemical technique and assay. Intern. Comm. Ullevål Hospital, Oslo, Norway 1973.
- FRANTZMAN, A. W., A. FLYNN and B. D. ARNESEN, Serum corticoid levels relative to handling stress in Alaska moose. *Canad. J. Zool.* 1975 53: 1424-1426.
- GLICK, S. M., J. ROTH, R. S. YALOW and S. A. BARSON, The regulation of growth hormone secretion. *Recent Progr. Hormone Res.* 1965 21: 241-270.
- HOVE, K. and A. K. BLOM, Plasma insulin and growth hormone in dairy cows, diurnal variation in relation to food intake and plasma sugar and acetone levels. *Acta endocr. (Kbh.)* 1973 73: 289-294.
- JOHNSTON, J. D. and R. B. BUCKLAND, Response of male Holstein calves from seven sires to four different stresses as measured by plasma corticoid levels. *Canad. J. Anim. Sci.* 1976 55: 777-781.
- NOBIL, R. L., The physiology of the adrenal cortex. In *The Hormones*, Eds. O. Finkelstein and K. V. Thimann, Vol. II Academic Press, New York, 1950, 65-180.
- NOBIL, R. L., The physiology of the adrenal cortex. I. *The Hormones*, Eds. O. Finkelstein and K. V. Thimann, Vol. III Academic Press, New York, 1955, 685-876.
- OLSSON, J. and A. TRENDEL, Exposure of cattle to controlled subzero temperature: growth hormone, glucose and free fatty acid concentrations in plasma. *Amer. J. Vet. Res.* 1973 34: 747-751.
- PIRELLA, B. A. and M. R. VICKERY, The distribution of cortisol and its rate of turnover in normal cold-stressed sheep. *J. Endocr.* 1972 55: 519-531.
- RAWSON, R. W., J. E. RALL and M. SONNENBERG, The chemistry and physiology of the thyroid. In *Hormones*, Eds. O. Finkelstein and K. V. Thimann, Vol. III, Academic Press, New York, 1955, 431-571.

Immobilizing the beta-adrenergic receptor blocker propranolol and the "immobilization-stress" response could also be blocked by adrenalectomy. An increase in CMRO₂ has been shown to occur during hypoxia (Berntman *et al.* 1977). In man considerable variations in CBF and CMRO₂ have been reported when repeated measurements were made in the patients during "resting conditions" (Lassen *et al.* 1977). To gain further knowledge about the factors affecting CMRO₂ and CBF (*i.e.* blood flow per weight of brain) we have in the present study investigated the effect of CO₂ and the effect of beta-adrenergic receptor blockage upon these variables. The conditions presently used are partly the same as those investigated by Berntman *et al.* (1978) using the Kety-Schmidt technique with inhalation of ¹³³Xe for determination of CBF. It is apparent from previously mentioned studies that the "resting level" for CMRO₂ and CBF may not be well defined as originally thought and therefore we have applied a technique which allows CBF-measurements at short intervals thus in testing the effect of various agents the animal itself may serve as its own control. This technique also has the advantage of giving more reliable results than the Kety-Schmidt technique (Eklöf *et al.* 1973) at the high CBF values encountered during hypercapnia.

Methods

rats weighing 200-300 g were used. The animals had free access to water and food pellets until used. Cerebral blood flow (CBF) and oxygen consumption (CMRO₂) were measured using the rapid and reliable method recently developed in our laboratory (Hertz *et al.* 1977). This extracranial ¹³³Xe injection technique was originally described in man by Hassel-Rasmussen *et al.* 1966. The animals were preoxygenated with halothane 0.8% in gas mixture of 20% N₂O/80% O₂, tracheotomized and artificially ventilated on an animal respirator (Braun, Melsungen). All extracranial lines from both carotid arteries are ligated including the pterygopalatine artery. A polyethylene catheter for injection of ¹³³Xenon was inserted in a retrograde fashion into the right external carotid artery. A catheter was placed at the carotid bifurcation to avoid obstruction of flow into the internal carotid artery as catheterized for continuous recording of mean arterial blood pressure (MABP) for measurement of pH_a, PaCO₂ and PaO₂ using microelectrodes (Radiometer Copenhagen). A catheter was also cannulated to enable blood transfusion and drug infusion. A small skin incision was made on the head and through a burr hole in the lambdoid process a cannula was inserted for sampling of arterial blood from the carotid sinus. Rectal temperature was kept close to 37°C (±0.5°C) by use of heating blanket. MABP was kept within ±10% of the basal value by transfusion of fresh donor blood. All wounds are infiltrated with lidocaine 1% to avoid pain after switching off halothane. At completion of the surgical preparation, each took 40-45 min, the animals were anesthetized with amyl chloride 20 mg/kg and halothane was switched off. At each measurement of CBF, 20 µl bolus of the radioactive, inert gas ¹³³Xenon dissolved in saline (NaCl, 0.9%, Acridolam) is injected into the internal carotid and the washout curve was obtained with a NaI(Tl) crystal (aperture 8 mm) placed over the head ipsilateral to the injection catheter. For determination of CMRO₂, arterial and cerebral venous blood was sampled in 25 µl Carlsberg pipettes 30 s after ¹³³Xenon injection and in these blood samples total oxygen concentration was measured by the polarographic method described by Borgström *et al.* (1974). CBF and CMRO₂ measurements in the different situations were made in duplicate and the values averaged.

Calculations

r was calculated from the initial slope of a semilog plot of the ¹³³Xenon washout clearance curves. The formula used is given by Olesen *et al.* (1971): $rCBF_{\text{min}} = \lambda \ln 10 \cdot D_0/100$ (ml/100 g/min) using

The effect of propranolol on cerebral oxygen consumption and blood flow in the rat measurements during normocapnia and hypercapnia

By

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Abstract

HEMMINGSEN R., M. M. HERTZ and D. I. BARRY *The effect of propranolol on cerebral oxygen consumption and blood flow in the rat measurements during normocapnia and hypercapnia* Acta physiol. scand. 1979 105, 274-281

The cerebral blood flow (CBF) and cerebral oxygen consumption ($CMRO_2$) in the rat during normocapnia and hypercapnia were investigated by means of the intraarterial ^{133}Xe injection technique. Measurements were performed during normocapnia and hypercapnia and the effect of propranolol upon CBF and $CMRO_2$ was studied. The CBF technique applied to rat yield reliable results even in high flow situations. A state period of only 10-15 s is all that is necessary to obtain the initial slope of the ^{133}Xe clearance from which CBF is calculated and measurements may be repeated within minutes. Hypercapnia caused an increase in $CMRO_2$ of 35% which confirms the findings of other investigators. The beta-adrenergic receptor blocker propranolol (2 mg per kg i.v.) prevented this increase and could eliminate the increase in $CMRO_2$ already induced. This indicates that CO_2 affects adrenergic mechanisms. Although propranolol eliminated the $CMRO_2$ response to hypercapnia, it only reduced the CBF response: this decrease in CBF and $CMRO_2$ response occurred probably because the beta-receptor blockade only eliminated the increase mediated through an increased $CMRO_2$ (cellular response) whereas a direct CO_2 effect upon arterioles (vascular response) persisted.

It has been generally considered that the cerebral rate of oxygen consumption ($CMRO_2$ "cerebral metabolic rate for oxygen" - ml O_2 100 g brain min^{-1}) could not increase above the "resting level" found in conscious individuals except during extreme conditions. Such conditions mentioned in the older literature include seizures (Schmidt *et al.* 1951), extreme anxiety (Kety 1952) and continuous i.v. infusion of epinephrine (King *et al.* 1953).

Recently however it has been shown that $CMRO_2$ may increase in unanesthetized rats during immobilization, "immobilization stress" (Carlsson *et al.* 1977) and during hypercapnia (Berntman *et al.* 1978). In both situations the $CMRO_2$ -increase could be prevented

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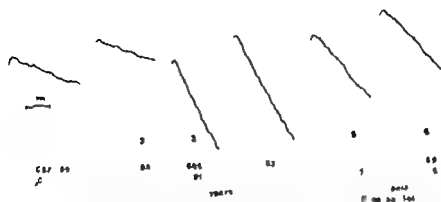


Fig. 3. Six reproducibly recorded ^{15}m Xenon wash-out curves which illustrate the reproducibility of CBF measurements. Measurements are from an animal of group A. Double measurements were made at 5 min interval during normocapnia, hypercapnia and during hypercapnia after administration of propranolol 2 mg/kg. I.V. PaCO_2 in mmHg; CBF is ml/100 g/min. Mean arterial blood pressure virtually unchanged.

When rats were given propranolol a slight decrease in MABP occurred, but all animals had an ABP above 130 mmHg which is normal for rats of this strain. Table II shows values of BF and CMRO_2 . When hypercapnia was induced CBF increased by 2.5 times the normocapnic value and when propranolol was given CBF decreased to 1.8 times the normocapnic value (cf. Table II). These changes were statistically significant. During hypercapnia CMRO_2 rose by 35% of the normocapnic value and after administration of propranolol CMRO_2 returned to the normocapnic value, i.e. the significantly higher CMRO_2 existing during hypercapnia was pharmacologically reduced to the initial level.

Group B

Values of PaCO_2 , PaO_2 , pH and MABP from this group are given in Table III. As in Group A hypercapnia was accompanied by the expected decrease in pH . MABP was kept actually unchanged after induction of hypercapnia. Table IV shows CBF and CMRO_2 . During hypercapnia led to an increase in CBF by 1.8 times the normocapnic value whereas no change in CMRO_2 occurred.

Table III. Group A. PaCO_2 , PaO_2 , pH and mean arterial blood pressure (MABP) in rats during normocapnia and during hypercapnia without and with propranolol 2 mg/kg I.V. Medians and 25-75 per centiles are given. N = 7.

	PaCO_2 (mmHg)	PaO_2 (mmHg)	pH	MABP (mmHg)
Normocapnia	36 (34-41)	121 (115-123)	7.40 (7.38-7.40)	150 (147-153)
Hypercapnia	81 (78-82)	120 (124-133)	7.14 (7.10-7.17)	158 (148-160)
Hypercapnia + Propranolol	33 (27-33)	108 (105-114)	7.89 (7.04-7.12)	136 (132-144)

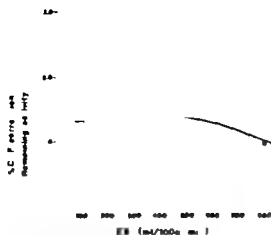


Fig. 1 Curve for correction for remaining activity from previous measurements. The actually measured CBF is found on the abscissa and then the corresponding ordinate-value shall be multiplied by the percentage of remaining activity to yield the correction factor to be added to the measured CBF-value.

tissue: blood partition coefficient for grey matter $\lambda_g = 0.87 \text{ ml/g}$. D_0 is the initial slope of the ascending non-residue clearance curve. The slope was obtained from the first 10–15 s of the curve.

A correction for the activity remaining from previous measurements was applied when this activity exceeded 2% of the peak height of the lineary washout curve. The correction was performed as described by Olesen *et al.* (1971) using the graph in Fig. 1. The correction thus rests on the assumption that the remaining activity stays constant during the period required for measurement of the initial slope and that the correction is proportional to the remaining activity when this constitutes 0–10 per cent of the peak activity remaining after the bolus injection. The corrections are therefore valid as long as the CBF of grey matter is several times higher than the CBF of white matter. In the present study no measurement was made until the remaining activity was less than 5% of the peak value.

CMRO_2 was calculated by multiplying the arteriovenous difference in oxygen concentration by the corrected CBF value.

Experimental group

3 experimental groups were studied. In each group the hypercapnia was induced by adding CO_2 to the inhaled gas mixture of 70% N_2O –30% O_2 . In group A and B propranolol (mg/kg diluted in 0.5 ml heparinized blood) was administered slowly over a period of 5 to 8 min (this being necessary to avoid a sudden decrease in MABP).

Group A (7 animals) After immobilization and removal of halothane, the rats were allowed to equilibrate for a period of 20 min before CBF and CMRO_2 were measured during normocapnia. Hypercapnia was induced (PaCO_2 adjusted to 80 mmHg) and 15–20 min later CBF and CMRO_2 were measured again. In maintaining hypercapnia, propranolol was administered and after 30 min the final CBF and CMRO_2 measurements made.

Group B (6 animals) In this group of animals propranolol was given immediately after halothane was switched off and the animals had been immobilized. After 30 min of normocapnia CBF and CMRO_2 were measured. Hypercapnia was induced and after 15–20 min CBF and CMRO_2 were measured again.

Group C (4 animals) After halothane was switched off CBF and CMRO_2 were measured in 4 animals after 20 min and 30 min of hypercapnia in order to investigate whether CBF and CMRO_2 changed during the intervening period.

Statistical differences were tested by non-parametric methods. Differences within groups were tested by Wilcoxon's test for paired data whereas differences between groups were tested by Mann-Whitney test for unpaired data.

Results

The method used for measurements of CBF is theoretically and practically well suited for repetitive measurements in both high flow and rather low flow situations (see discussion). The reproducibility of measurements is illustrated in Fig. 2.

Group A

Values of PaCO_2 , PaO_2 , pHa and MABP are given in Table I. As expected an increase in PaCO_2 to about 80 mmHg was accompanied by a decrease in pHa to about 7.1. When the

in). After 30 min of hypercapnia (PaCO_2 89 mmHg) CBF was 430 ml/100 g/min (range 417-448 ml/100 g/min) and CMRO₂ was 10.5 ml/100 g/min (range 9.7-12.5 ml/100 g/min). MABP and pH_a were unchanged. CBF and CMRO₂ were thus unchanged by 30 min of hypercapnia.

Discussion

The perfusion coefficient (blood flow per unit weight of tissue) is (normally) far greater in the grey matter than in the white matter and since white matter constitutes only a rather small part of the rat's brain (Nilsson and Siesjö 1976), the major part of the intracarotidly injected ^{86}Kr will initially be located in the grey matter. Consequently the initial slope of the recorded ^{86}Kr clearance curve will predominantly reflect the perfusion coefficient of the grey matter (extracerebral contamination was avoided by ligation of the extracerebral branches of the carotid artery). Venous blood collected from the torcular sinus will mainly be saturated by blood perfusing grey matter and the arterio-venous oxygen concentration difference calculated from the oxygen concentration of such venous blood should be close to the $a-v$ difference for blood perfusing grey matter only. It follows that a product of a related $a-v$ difference and the corresponding calculated perfusion coefficient should give the rate of oxygen consumption per unit weight of grey (cortical?) matter with good approximation.

In spite of the close theoretical relationship (Lassen 1976) between the Kety-Schmidt method (modified for rat by Ekblom *et al.* 1973) and the intracarotid injection method there are several reasons rendering the latter method preferable in rat studies. 1) Only a 10-15% reduction of constant cerebral blood flow and flow distribution is necessary to obtain the initial slope, and 2) measurements can be repeated within few minutes thus enabling a more dynamic approach to the study of CBF and CMRO₂. 3) In the rat very high flow values may occur and in such situations CBF can easily be calculated from the initial slope whereas calculation according to the Kety-Schmidt principle rests heavily upon determination of one or two venous concentrations within the initial period of desaturation when the concentration changes quite rapidly with time.

The discussion of the CBF and CMRO₂ results will be separated in 3 parts.

- 1) The effect of hypercapnia upon CMRO₂.
- 2) The relation between CBF and CMRO₂.
- 3) Resting state values of CBF and CMRO₂.

All the induction of hypercapnia (PaCO_2 about 80 mmHg) resulted in a 35% increase in CMRO₂, which is consistent with the 22% difference between normocapnic and hypercapnic rats found by Bernstein *et al.* (1978). In an earlier study (Ekblom *et al.* 1973) no increase in CMRO₂ was measured but their CBF-technique is not suited for measurement of the very high CBF-values occurring during hypercapnia. In both studies animals were anaesthetized with 70% N_2O and O_2 .

The CO_2 -induced increase in CMRO₂ was prevented by the beta-adrenergic receptor blocker propranolol and an increase already induced could be eliminated by propranolol. The decrease in CMRO₂ in the hypercapnic animals after administration of propranolol

TABLE II *Group A* Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMRO₂) at normocapnia and at hypercapnia without and with propranolol 2 mg/kg I.V. Median 25-75 percentiles are given. N=7

	CBF (ml/100 g/min)	CMRO ₂ (ml/100 g/min)	Statistics (within group)
Normocapnia (N)	103 (99-135)	13.1 (12.4-14.1)	CBF N/H p < 0.05 N/H p < 0.05 H/H p < 0.05
Hypercapnia (H)	361 (313-461)	17.7 (14.8-19.2)	CMRO ₂ N/H p < 0.05 H/H p < 0.05
Hypercapnia + propranolol (H/P)	278 (224-301)	12.9 (10.1-15.3)	

TABLE III *Group B* PaCO₂, PaO₂, pH and mean arterial blood pressure (MABP) in normocapnia and hypercapnia in animals given propranolol 2 mg/kg I.V. Median and 25-75 percentiles are given. N=6.

	PaCO ₂ (mmHg)	PaO ₂ (mmHg)	pH	MABP (mmHg)
Normocapnia + propranolol	35 (34-36)	123 (117-126)	7.45 (7.44-7.45)	148 (147-149)
Hypercapnia + propranolol	80 (76-85)	134 (133-137)	7.10 (7.09-7.12)	145 (136-150)

Comparison between group A and B

Both CBF and CMRO₂ during normocapnia were lower in the propranolol treated group as compared to the untreated group A ($p < 0.05$). This was also the case during hypercapnia when propranolol treated animals of group B were compared to the still untreated animals of group A. (For CBF $p < 0.05$ for CMRO₂ $0.05 < p < 0.10$). The median CMRO₂ in untreated, hypercapnic animals of group A was 65% higher than the corresponding value in the treated group B.

There were no statistical differences in CBF or CMRO₂ between hypercapnic animals treated with propranolol before (group A) and after (group B) induction of hypercapnia although the values in group A tended to be higher.

Group C

After 20 min of hypercapnia (PaCO₂ ~85 mmHg) CBF was 463 ml/100 g/min (quartiles 430-528 ml/100 g/min) and CMRO₂ was 10.2 ml/100 g/min (quartiles 9.4-13.7 ml/100 g/min).

TABLE IV *Group B* Cerebral blood flow (CBF) and cerebral metabolic rate for oxygen (CMRO₂) during normocapnia and hypercapnia in animals given propranolol 2 mg/kg I.V. Median 25-75 percentiles are given. N=6.

	CBF (ml/100 g/min)	CMRO ₂ (ml/100 g/min)	Statistics (within group)
Normocapnia (N) + propranolol	73 (71-73)	10.8 (10.2-11.3)	CBF N/H p < 0.05
Hypercapnia (H) + propranolol	207 (164-253)	10.7 (9.2-11.3)	CMRO ₂ ns

After 50 min of hypercapnia ($\text{PaCO}_2 = 89 \text{ mmHg}$) CBF was 430 ml/100 g/min (range $417-448 \text{ ml/100 g/min}$) and CMRO_2 was $10.5 \text{ ml/100 g/min}$ (quartiles $9.7-12.5 \text{ ml/100 g/min}$). MABP and pH_a were unchanged. CBF and CMRO_2 were thus unchanged after 50 min of hypercapnia.

Discussion

The perfusion coefficient (blood flow per unit weight of tissue) is (normally) far greater in grey matter than in the white matter and since white matter constitutes only a rather small part of the rat's brain (Nilsson and Sjöström 1976), the major part of the intracarotidly injected ^{133}Xe will initially be located in the grey matter. Consequently the initial slope of recorded ^{133}Xe clearance curve will predominantly reflect the perfusion coefficient of grey matter (extracerebral contamination was avoided by ligation of the extracerebral branches of the carotid artery). Venous blood collected from the torcular sinus will mainly be saturated by blood perfusing grey matter and the arterio-venous oxygen concentration difference calculated from the oxygen concentration of such venous blood should be close to the $a-v$ difference for blood perfusing grey matter only. It follows that a product of a constant $a-v$ difference and the corresponding calculated perfusion coefficient should be the rate of oxygen consumption per unit weight of grey (cortical?) matter with good approximation.

In spite of the close theoretical relationship (Lassen 1976) between the Kety-Schmidt method (modified for rat by Eklöf *et al.* 1973) and the intracarotid injection method there are several reasons rendering the latter method preferable in rat studies: 1) Only a 10-15% reduction of constant cerebral blood flow and flow distribution is necessary to obtain the initial slope, and 2) measurements can be repeated within few minutes thus enabling a more dynamic approach to the study of CBF and CMRO_2 . 3) In the rat very high flow values may occur and in such situations CBF can easily be calculated from the initial slope whereas calculation according to the Kety-Schmidt principle rests heavily upon determination of one or two arterial concentrations within the initial period of desaturation when the concentration changes quite rapidly with time.

The discussion of the CBF and CMRO_2 results will be separated in 3 parts.

- 1) The effect of hypercapnia upon CMRO_2 .
- 2) The relation between CBF and CMRO_2 .
- 3) "Resting state values" of CBF and CMRO_2 .

At induction of hypercapnia (PaCO_2 about 80 mmHg) resulted in a 35% increase in CMRO_2 which is consistent with the 22% difference between normocapnic and hypercapnic rats found by Bernstein *et al.* (1978). In an earlier study (Eklöf *et al.* 1973) no increase in CMRO_2 as measured but their CBF-technique is not suited for measurement of the very high CBF-values occurring during hypercapnia. In both studies animals were anaesthetized with N_2O 30% O_2 .

The CO_2 -induced increase in CMRO_2 was prevented by the beta-adrenergic receptor blocker propranolol and an increase already induced could be eliminated by propranolol. The decrease in CMRO_2 in the hypercapnic animals after administration of propranolol

was not due to a simple time-effect as no decrease was observed in 4 animals (group C) hypercapnic for the same period of time without receiving propranolol.

The ability of propranolol to block the CMRO increase during hypercapnia indicates that CO₂ affects adrenergic mechanisms. Adrenalectomy does not prevent the CMRO increase caused by hypercapnia (Berniman *et al* 1978) and hence CO₂ must be exerting its CMRO increasing effect centrally. The finding that CO₂ increases the electrical activity of cerebral cortex as measured by EEG in sleeping man (Bilow 1963) also points to an activating effect of CO₂ on the brain as does the increased rate of formation of arylamines found in the brain during hypercapnia (Proulx *et al* 1977). Carlsson *et al* (1977) and MacKenzie *et al* (1976) have shown in baboons (normocapnia) that a CMRO₂-increase of the same order of magnitude (23%) can be measured during reserpine induced release of endogenous cerebral norepinephrine, whereas administration of propranolol was followed by a decrease in CMRO₂ as compared to pretreatment value.

It should be emphasized that the effect of CO₂ upon CMRO is by no means fully elucidated. In the 4 animals of group C the CMRO₂ values observed (during hypercapnia) did not differ significantly from those obtained in animals of group A in the normocapnic period. This may indicate interindividual differences in brain reactivity to CO₂.

4d 2) After administration of propranolol a dissociation between CBF and CMRO₂ response to changing PaCO₂ was revealed. Propranolol eliminated the CMRO₂-response to hypercapnia whereas the CBF response was only moderately reduced. The latter finding confirms earlier results from baboon (MacKenzie *et al* 1976, Aoyagi *et al* 1976) and man (Berniman *et al* 1978).

It seems most probable that the dissociation of CBF and CMRO₂ response to hypercapnia after propranolol was established because the beta receptor blockage only eliminated a CBF increase mediated through an increased CMRO₂ (cellular response) whereas a direct CO₂ effect upon the arterioles (vascular response) persisted. This direct effect was probably mediated through an increased hydrogen ion concentration (see Severinghaus and Lein 1967). The beta-adrenergic receptors responsible for the metabolic CO₂-response are probably localized in the cerebral cortical parenchyma. beta-receptors have been shown to exist in this location whereas no beta-receptors were seen in the brain vessel walls of the baboon when using a fluorescence technique (Melamed *et al* 1977). In relation to this it is important to know that propranolol easily crosses the blood brain barrier (Olesen *et al* 1978).

4d 3) During "resting state conditions" (normocapnia, N₂O anaesthesia) the propranolol treated animals (group B) had a significantly lower CMRO₂ than had the untreated animals (group A). The CMRO₂ just above 10 ml/100 g/min obtained in group B was close to the value obtained by Carlsson *et al* (1977) in propranolol treated unanaesthetized rats ("mobilization stress") and this may reflect an ability of beta-adrenergic receptor blockade to affect adrenergic "tonus" upon CMRO₂ and hence to diminish interindividual variation in CMRO₂ in unanaesthetized and lightly anaesthetized animals.

References

- Aoyagi, M., V. D. Deshmukh, J. E. Meyer, Y. Kawamura and Y. Tagashira, Effect of beta-adrenergic blockade with propranolol on cerebral blood flow autoregulation and CO₂ responsiveness. *Stroke* 1978, 7: 291-295.

- MAN, L., C. CARLSSON and B. K. SUND, Cerebral blood flow and cerebral metabolic rate in arterial occlusion and ischaemia. *Acta neural. scand.* 1977 Suppl. 33 56, 96-97
- MAN, L., M. DANLON and B. K. SUND, Influence of moderate and extreme hypercapnia on cerebral blood flow and oxygen consumption in the rat brain. *Anesthesiology* 1978. (submitted for publication).
- MAN, L., M. HILGREN, L. LARSEN and U. PONTIN, Polarographic determination of total oxygen content in small blood samples. *Scand. J. clin. Lab. Invest.* 1974, 34 375-380.
- MAN, L., F. Respiration and metabolism in man. *Acta physiol. scand.* 1963, 59 Suppl. 209
- MAN, L., M. HILGREN, A. E. KASSEL and B. K. SUND, A catecholamine-mediated increase in cerebral oxygen uptake during ischaemia in rats. *Brain Res.* 1977 119 223-231
- MAN, L., T. HOLM, M. LUNDQVIST and B. K. SUND, Effect of hypercapnia and hypocapnia on tyrosine and tyrosine hydroxylation in rat brain. *Acta physiol. scand.* 1977 99 503-509
- MAN, L., N. A. LARSEN, L. NILSSON, K. NORRMAN and B. K. SUND, Blood flow and metabolic rate for glucose in the cerebral cortex of the rat. *Acta physiol. scand.* 1973, 88 587-589.
- MAN, L., M. R. HANSEN and T. G. BOLWIG, Rapid and repetitive measurements of blood flow and oxygen consumption in the rat brain using intraarterial Xenon injection. *Acta physiol. scand.* 1977 101 1-10.
- MAN, L., K. E. SVENSSON and N. A. LARSEN, A regional cerebral blood flow in man determined by intraarterial injection of radioactive inert gas. *Circulation Res.* 1966 18 37-47
- MAN, L., D. Problems of Cerebral Circulation. New York: Macy 1952, pp. 26-40.
- MAN, L., D. L. SODERLUND and R. L. WICKSTEAD, The effect of α -phenylephrine and α -nor-epinephrine upon cerebral circulation and metabolism in man. *J. clin. Invest.* 1952, 31 273-279
- MAN, L., N. A. Methods for measurement of cerebral blood flow in man. *Med. Progr. Technol.* 1976, 3, 4-10.
- MAN, L., P. E. ROLAND, B. LARSEN, E. MELAND and K. SUND, Mapping of human cerebral functions. Study of the regional cerebral blood flow pattern during rest, its reproducibility and the activation seen during basic sensory and motor functions. *Acta neural. scand.* 1977 56, Suppl. 64 262-263
- MAN, L., E. T. J. MCCULLOCK and A. H. HANSEN, Influence of endogenous nor-epinephrine on cerebral blood flow and metabolism. *Amer. J. Physiol.* 1976, 231 489-494.
- MAN, L., D. ATLAS and M. LARSEN, Direct evidence for absence of beta-adrenergic receptors in rat cerebral vessels. *Stroke* 1977 8 281-283.
- MAN, L. and B. K. SUND, A method for determining blood flow and oxygen consumption in the rat brain. *Acta physiol. scand.* 1976, 96 72-82.
- MAN, L., O. B. FALSTAD and N. A. LARSEN, Regional cerebral blood flow in man determined by the initial slope of the clearance of intra-arterially injected ^{133}Xe . *Stroke* 1971 2 519-540.
- MAN, L., K. HOLM and M. M. HARTZ, Isoprenaline and propranolol. Ability to cross the blood-brain barrier and effects on cerebral circulation in man. *Stroke* 1978, 9 344-353
- MAN, L., G. Y. A. CARLSSON and M. A. M. GOWAT, The effect of CO_2 on monoamine metabolism in rat brain. *Acta Pharmacol.* 1977 301 11-15.
- MAN, L., C. F. S. S. KITTY and H. H. PENNIE, Glucose metabolism of the brain of the monkey. *Amer. J. Physiol.* 1945 143, 33-52.
- MAN, L., J. W. and N. A. LARSEN, *Serp* hypocapnia to separate arterial from tissue PCO_2 in the regulation of cerebral blood flow. *Circulation Res.* 1967 20, 273-278.

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References

- Aoyagi, M., V. D. Deshmukh, J. S. Meyer, Y. Kawamura and Y. Tagashira, Effect of beta-adrenergic blockade with propranolol on cerebral blood flow autoregulation and CO₂ responsiveness. *Stroke* 1978, 7: 291-295.

code strength (Folkow *et al.* 1971 b). However the relationships between arterial pressure (P_A) and flow (Q), obtained during perfusion with plasma substitute at maximal filtration in the mentioned study gave the impression that the SHR renal resistance vessels were structurally somewhat widened compared with the NCR ones, in contrast to resistance in other circuits. Recently Collis and Vanhoutte (1977), in a similar connection but using a colloid-free perfusate, observed no real difference between the SHR and NCR renal vascular beds in this latter respect.

His results concerning total renal resistance are in apparent contrast to recent findings (Folkow *et al.* 1977), which strongly suggest a considerable, structurally based increase of resistance between the pre- and postglomerular resistances in adult SHR, a difference to NCR was barely traceable in early borderline hypertensive age. Thus, when P_A was from very low levels gradually elevated to parallel-perfused maximally vasodilated SHR and NCR rats, glomerular filtration (GF) started in SHR first at a P_A level that was 30–40 mmHg higher than in NCR. Further at still higher P_A elevations the P_A -GF relationship for the kidneys was throughout shifted some 30–40 mmHg along the P_A abscissa to the right in NCR ones. Likewise, if ureter pressures were equally increased in similarly perfused SHR and NCR kidneys, GF ceased at a much lower ureter pressure level in SHR than in NCR (Gottberg *et al.* 1976). Such findings can be explained only by a considerable increase of structurally determined pre/postglomerular resistance ratio in established SHR hypertension, while glomerular filtration capacity was not yet appreciably reduced to judge from similarity of the P_A -GF relationships in SHR and NCR (Folkow *et al.* 1977).

A question arises how such apparently contradictory results concerning total versus extra-segmental renal resistances may be explained. A clue is given by the observation (Gottberg *et al.* 1976) that tissue pressure (P_t) was consistently much lower in SHR than in NCR kidneys at equal P_A levels, further that P_t increased far less in the SHR kidneys at the same P_A increments. It is known that P_t in kidneys mainly reflects the tubular distension fluid, caused by the glomerular filtration. The higher the GF rate, and/or the lower the tubular reabsorption at given resistance characteristics of the tubular system, the higher the tubular pressure. For such reasons renal P_t increases will be particularly marked when glomerular filtration is extensive (e.g. at low perfusate colloid osmotic pressures as in Collis and Vanhoutte's study) or/and when tubular reabsorption is greatly reduced (e.g. by perfusate bag as in the study by Folkow *et al.* 1977). Since active preglomerular autoregulation of renal flow is eliminated by maximal vasodilatation during such artificial perfusions, P_A is bound to be associated with considerable and largely proportional P_t elevation. However a particularly strong renal 'passive' autoregulation of flow is thereby introduced (Marshall 1958, Folkow and Langston 1964) because the true pressure head approximates P_t rather than P_A alone. A structurally increased pre/postglomerular resistance ratio in SHR then implies that P_t rises will here be less pronounced than in NCR. In other words, the true pressure head, $P_t - P_A$ is at equal P_A levels correspondingly higher in SHR than in NCR.

For such reasons the true renal resistance to flow in SHR and NCR cannot be properly judged by the P_A - Q relationships alone as long as glomerular filtration occurs, implying that the results of both Folkow *et al.* (1971 b) and of Collis and Vanhoutte (1977) are in this

Apparent and true vascular resistances to flow in SHR and NCR kidneys as related to the pre/postglomerular resistance ratio

By

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Abstract

GÖTHBERG G., S LUNDIN S. E. RICKSTEN and B. FOLKOW. *Apparent and true vascular resistances to flow in SHR and NCR kidneys as related to the pre/postglomerular resistance ratio*. Acta physiol. scand. 1979 105: 282-294.

In maximally vasodilated SHR and NCR kidneys, perfused with filtrable and non-filtrable polystyrenes, analyses have been performed concerning the effect of average renal tissue pressure (P_t) elevation which occurs upon glomerular filtration and causes passive autoregulation of flow. The results illustrate the importance of distinguishing between apparent ($(P_A - P_V)/\text{flow}$) and true ($(P_A - P_G)/\text{flow}$) renal vascular flow. This is particularly so when the compared SHR and NCR renal vascular beds differ not only in total renal resistance but also concerning the structurally determined ratio between the pre- and postglomerular resistances. The combined results show that this ratio is considerably elevated in adult SHR kidneys because of structural vascular changes, which is perhaps the most efficient way of reacting to the term barostatic function of the kidneys in hypertension. It is also illustrated how the altered pre/postglomerular ratio in SHR in combination with P_G rises can so markedly distort the results of apparent flow comparisons of SHR and NCR renal vascular beds as to give entirely misleading results with underestimations of the structurally based vascular hyperactivity of the preglomerular bed in SHR.

Among systemic circuits the renal vascular bed is of particular interest in hypertension because it affects so intimately the water-salt equilibrium (cf. Guyton *et al.* 1974), by contributing to the overall increase of resistance. It is known from many studies (e.g. Folkow *et al.* 1973; Folkow 1975; Norell, Hallbjörk and Hjalmarsson 1978) that systemic resistance vessels almost from the start of hypertension develop a proportional adaptive thickening associated with a structural luminal narrowing, a type of structural adaptation largely confined to the pressure-exposed precapillary resistance vessels where, as an amplifying and strengthening lever for active changes in smooth muscle tone (Folkow *et al.* 1974). Also the renal resistance vessels appear to exhibit a considerable thickening in hypertension, to judge from hemodynamic comparisons of perfused kidneys from SHR and normotensive controls (NCR) and here reflected as an increased slope of the dose response resistance curve to pressor agents, associated with an elevated

are closely similar. As measured with an Ostwald viscometer, the viscosity of kerosene was 35^m less than that of 2% dextran-Tyrode solution at equal temperatures. Therefore the kerosene flow values are multiplied by 1.35 in order to provide directly comparable pressure-flow curves for the two perfusates prior to their viscosity. These curves then coincided well at pressures below the levels where glomerular filtration starts.

In experiments the approximate intrarenal tissue pressure was measured in the dextran-Tyrode perfused kidneys by a series of cannulae of 0.3 mm diameter which were inserted to identical depths into the renal cortex. It should be realized, however, that such local pressures will only exceptionally be representative of the true average intrarenal pressure level, and were therefore mainly used for general orientation in events.

Results

Arterial pressures in the awake NCR and SHR differed about 30 per cent, this difference being slightly lower (about 23 per cent) in the subgroup used for kerosene perfusions, because NCR here showed slightly higher pressures than is usual for NCR.

In dextran-Tyrode perfused kidneys immediate increases of renal flow from a low, constantly held level caused a two-phasic P_A increase: first an initial prompt rise, followed by a second phase where P_A increased progressively and gradually levelled off after 15-30 sec (Fig. 1). This second phase of P_A rise was always, as illustrated in this Figure, more marked in NCR than in SHR. As flow was then constant, the second phase of the P_A rise reflects a progressive increase of resistance which, however, cannot represent any autoregulatory response since the vessels remained completely relaxed due to high concentrations of vasodilator agents and the low tissue temperature. In this situation a 50% proportional increase of glomerular filtration (GF) will occur progressively dilating the tubules and increasing pressure within them. Some accumulation of fluid might also in the renal interstitium in case filtration occurs across the tubular capillaries as

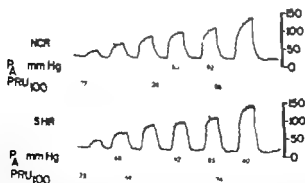


Fig. 1. Changes in arterial pressure (P_A) during paired perfusion with dextran-Tyrode solution of different rates of osmotically unclotted, deoxygenated kidneys from 3-4 month old SHR and NCR. When the increases in flow are induced, here lasting only 10 sec, there is a two-phasic P_A elevation: an initial phase with the sudden flow increase followed by a more gradual phase that usually levels off after 15-30 sec. As flow is constant during this second phase of P_A rise, it reflects a progressive increase of resistance within the kidneys, which must be ascribed to passive phenomena since the renal vessels are fully relaxed and unresponsive (passive autoregulation). The vertical dotted line roughly separates the phases and the deduced figures for renal resistance. Flow given as PRU values, therefore apparently reflects the truly vascular resistances present before passive autoregulation sets in. Note that the phase of passive autoregulation is more pronounced in NCR than in SHR.

particular respect misleading. The presence of passive autoregulation will obscure renal resistances in both NCR and SHR and particularly so in the NCR kidneys proportion to their lower pre/postglomerular resistance ratio. The aim of the present study is therefore to find out the true levels of total vascular resistance proper in unobstructed dilated SHR and NCR kidneys, thereby revealing also the extent to which P increases in the P_A - Q relationships during artificial perfusion and glomerular filtration. From the data and those by Folkow *et al* (1977) it would be possible to deduce more precisely the pre/postglomerular resistance ratio as well as the postglomerular resistance ratio when the SHR and NCR renal vascular beds are determined only by their structural dimensions. First then the nature, extent and hemodynamic significance of the structural vascular adaptation occurring in the SHR kidneys along with the development of hypertension can be fully understood. It should here be emphasized that a sustained increase in the pre/postglomerular resistance ratio represents perhaps the most efficient way of controlling the renal long term barostat function in hypertension (*cf* Guyton *et al* 1974).

Methods

20 matched pairs of male SHR and NCR at the age of 3-4 months were used. During light ether anesthesia the caudal artery of the two animals was cannulated with polyethylene catheters and mean arterial pressure (P_A) was measured after recovery from their anesthesia. Final anesthesia for the surgical preparation was induced by an intra-arterial infusion of Nembutal, 3-4 mg/100 g b.w.

In identical and largely simultaneous preparations a pair of SHR and NCR was entered by a midline abdominal incision. The abdominal aorta was freed for about 10 mm distal and 5 mm proximal to the renal arteries, great care being taken to ligate all branches along this aortic section except the two renal arteries and the superior mesenteric artery. In both animals the kidneys were then cautiously decapsulated and a catheter, directed into the renal arteries, was inserted in the distal aortic part after ligation of the proximal part of the same part of the aorta. The two kidneys with oxygenated 10 per cent dextran (Macrodex) in Tyrode solution were immediately started in both animals. The animals were killed and the proximal end of the aortic catheter was cannulated for P_A recording during the artificial renal perfusion in both animals. Once this procedure was completed, also the proximal aortic end was cannulated between the proximal ligation and the right renal artery and through this catheter the right kidneys of both animals could be perfused separately with the same oil kerosene after that a temporary ligature had been placed around the aorta between the two renal arteries. Thus, the right kidneys of the two animals were perfused in parallel with mineral oil and the left kidneys also in parallel, with oxygenated Tyrode solution containing 2% dextran.

To secure maximal vasodilatation nitroprusside sodium was dissolved in the 2% dextran-Tyrode solution, to give 0.8 mMol concentration. As the kerosene by itself relaxes the vasculature completely according to Wüsthoff, no vasodilating agent was used for the right kidneys. Both perfusions were kept at room temperature (20°C) to minimize active rectal reabsorption in the two left kidneys (*cf* Folkow *et al* 1977). The mineral oil kerosene earlier used by Wüsthoff (1958) for determining the true vascular resistance of the kidneys, is for such purposes suitable as it stays strictly within the renal vascular bed. Thereby glomerular filtration is eliminated, and with that largely the whole passive autoregulatory process in the right SHR and NCR kidneys, while the left ones will have their pressure-flow relations affected by passive autoregulation.

The pressure-flow relationship of the oil perfused kidneys was determined by repeated shifts in flow over a wide range during about 15 min. The same procedure was then performed concerning the dextran-Tyrode perfused kidneys of the two animals after that the right renal artery had been ligated, the temporary ligature between the renal arteries removed and the proximal catheter clamped.

The flow values both in the kerosene perfused right kidneys and the dextran-Tyrode perfused left kidneys were then calculated per g dry weight of the dextran-Tyrode perfused kidney as the dry weights of the kerosene-perfused kidneys are unreliable because the intravascular oil contents may not be completely eliminated in the heating chamber. The dry weights are for this purpose preferable since the dextran-Tyrode perfusion causes some renal edema which may differently affect the NCR and SHR kidneys. Experiments on fully intact SHR and NCR kidneys have shown that their relationships between renal flow and

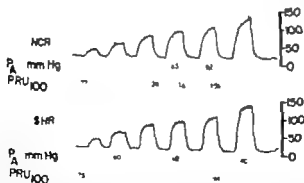
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To experimentally approximate intrarenal tissue pressure was measured in the dextran-Tyrosine kidneys by way of cannulas of 0.3 mm diameter which were inserted to identical depths into the cortex. It should be realized, however, that such local pressures will only exceptionally be representative of the true average intrarenal pressure level, and were therefore mainly used for general orientation.

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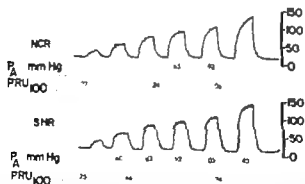
are closely similar. As measured with an Ostwald viscometer the viscosity of kerosene was 111 that of 2% dextran-Tyrode solution at equal temperatures. Therefore the kerosene flow values are multiplied by 1.35 in order to provide directly comparable pressure-flow curves for the two perfusates. Let us then discuss these curves. These curves then coincided well at pressures below the levels where glomerular flow starts.

In experiments the approximate intrarenal "tissue pressure" was measured in the dextran-Tyrode kidneys by way of cannulae of 0.3 mm diameter which were inserted to identical depths into the cortex. It should be realized, however, that such local pressures will only exceptionally be representative of the average intrarenal pressure level, and were therefore mainly used for general orientation events.

Results

Renal pressures in the awake NCR and SHR differed about 30 per cent, this difference being slightly lower (about 23 per cent) in the subgroup used for kerosene perfusions, because NCR here showed slightly higher pressures than is usual for NCR.

In dextran-Tyrode perfused kidneys immediate increases of renal flow from a low, constantly held level caused a two-phasic P_A increase, first an initial prompt rise, followed by a second phase where P_A increased progressively and gradually leveled off after (Fig. 1). This second phase of P_A rise was always, as illustrated in this Figure, more marked in NCR than in SHR. As flow was then constant, the second phase of the P_A rise reflects a progressive increase of resistance which, however, cannot represent any autoregulatory response since the vessels remained completely relaxed due to high dosages of vasodilator agents and the low tissue temperature. In this situation a proportional increase of glomerular filtration (GF) will occur progressively distal to the tubules and increasing pressure within them. Some accumulation of fluid might also be in the renal interstitium in case filtration occurs across the tubular capillaries as



Changes in arterial pressure (P_A) during paired perfusion with dextran-Tyrode solution at different rates of renally induced, decapsulated kidneys from 3-4 month old SHR and NCR. When increases in flow are induced, here lasting only 10 sec, there is a two-phasic P_A elevation; an initial phase with the sudden flow increase followed by a more gradual phase the usually levels off first. As flow is constant during the second phase of P_A rise, it reflects progressive increase of resistance (that the kidneys, both must be ascribed to passive phenomena since the renal vessels are fully relaxed and unresponsive ("passive autoregulation"). The vertical dotted line roughly separates the phenomenon and the deduced figures for renal resistance to flow given as PRU values, therefore apparently reflects the truly vascular resistances present before passive autoregulation sets in. Note that some of passive autoregulation is more pronounced in NCR than in SHR.

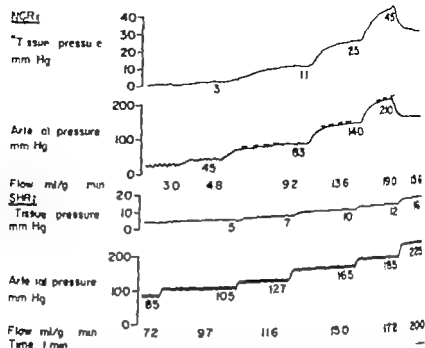
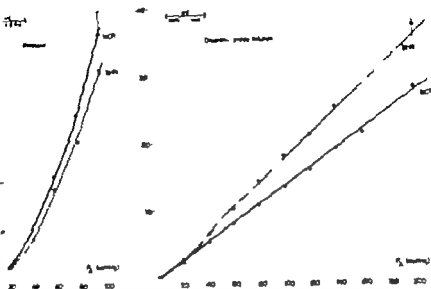


Fig. 2. Recordings of arterial pressure (P_A) and intrarenal tissue pressure (P_i), measured with thin cannulas inserted in identical cortical sites in pair-perfused decapitated SHR and NCR kidneys otherwise arranged as described in Fig. 1. This type of P_i recording, reflecting mainly the tubular pressure at the cannula site, shows semiquantitatively how P_i changes with P_A (though it does not necessarily reflect the average P_i level within the whole organ). However, the recordings serve to illustrate how P_i changes were consistently much smaller in SHR than in NCR for given changes in P_A .

Consequently the renal tissue pressure P_i will increase and largely in proportion to the rise in intratubular pressure. This tends to increasingly compress the low-pressure part of the renal vasculature, hence causing a progressive rise in renal resistance in which in reality is rather due to the fact that the true pressure head for vascular perfusion equals $P_A - P_i$. This passive autoregulatory influence of a P_i rise, which mainly opposes the enhanced GFR and tubular distension as P_A increases, thus produces an apparent rise in total renal vascular resistance that will not appear in case transcapillary fluid transport is hindered. In some experiments, where P_i was estimated by thin cannulas inserted in the parenchyma of the SHR and NCR kidneys in as identical portions as possible, it was observed that stepwise P_A increases always produced greater P_i increases in NCR, i.e. more pronounced GFR increases in the normotensive kidney for given P_A increments (Fig. 1).

However, this mode of estimating local intrarenal P_i may not with any accuracy reflect the average P_i changes within the kidney and their precise interference with renal blood flow and therefore it provides only semiquantitative information. In order to better quantify the differences in extent of passive autoregulation in the SHR and NCR kidneys, the P_A -Q relationships were determined over a wide range where flow/g wet kidney weight was plotted against the 'passively autoregulated' P_A levels for both SHR and NCR (Fig. 2, right part). It is seen from the Figure that the apparent total renal resistance to flow is around 25 per cent higher in the NCR kidneys at all P_A levels high enough to allow glomerular filtration. These P_A -Q relationships should be compared with those obtained



Left panel: 7 pairs of decapitated, maximally vasodilated kidneys from 3-4 month old SHR and NCR rats perfused with the non-filterable constant oil kerosene at room temperature, thus eliminating both active and passive autoregulatory processes (Weigelt 1958). Note the convexity towards the pressure axis reflecting the characteristic distensibility of resistance vessels whose fully relaxed, not further distally distended, truly vascular resistance to flow is moderately higher in SHR per g of renal tissue, a difference from NCR being significant ($p < 0.05$) at higher pressure levels.

Right: Left kidneys from the same pair of SHR and NCR rats here perfused with 2% Dextran in solution at room temperature and during maximal vasodilatation. Here glomerular filtration starts at low P_A levels and active reabsorption is maintained by cooling, renal tissue perfusion rises rapidly at elevations. The II data are represented above reached when 'passive autoregulation' is fully set. Since the viscosity of kerosene is 35% higher than that of 2% dextran-Tyrosine solution, the data in the left panel are corrected for this difference. Note how the pressure-flow curves for the two perfusions are greatly depressed towards the pressure axis once filtration starts, further that the NCR curves are now reversed.

g kerosene perfusion where passive autoregulatory influences caused by transcapillary transfer are eliminated. The P -flow relationships for kerosene are presented in the left panel of Fig. 3, but corrected for the difference in viscosity between kerosene and dextran-Tyrosine solution. It is then revealed that the true vascular resistance to flow is slightly higher (and 15 per cent) in the SHR kidneys than in the NCR ones. Moreover the P_A - Q relationships are now characteristically convex towards the pressure axis, which is typical of resistance vessels in general when both active and passive autoregulatory phenomena are relaxed, thus revealing their wall distensibility (Folkow and Löfving 1956, Hallock, Lippert and Weiss 1974).

Assuming that the vascular beds of the right and left kidneys have, on an average, equal perfusions in both NCR and SHR, the two sets of P -flow relationships in the left and right panels of Fig. 3 can be used to deduce the increases in P along with P_A elevations for the left and NCR kidneys, when plotted together (Fig. 4 left panel). These P increases are of interest since they approximately reflect the extent of glomerular filtration in the two

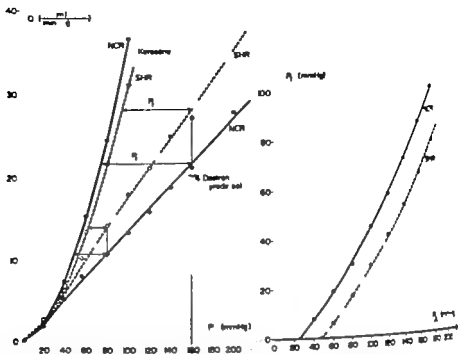


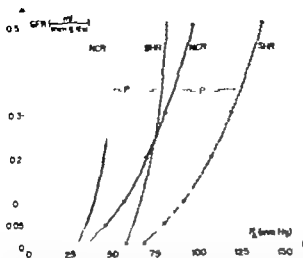
Fig. 4. *Left panel* The two pairs of pressure-flow curves from Fig. 3 are here plotted on the same scale for deductions of the average changes in renal tissue-pressure (P_t) from the difference in P_A at perfusion with dextran-Tyrode and kerosene as perfusates.

Right panel The deduced P_G level for given increases in P_A which are always higher in NCR than in SHR ones suggesting higher pre/postglomerular resistance ratio in SHR. It can hardly be a reduced glomerular filtration capacity in SHR since the $P_A - P_G$ relationships should then not be so

kidneys, if it is assumed that intratubular resistance to flow and degree of tubular reabsorption are largely equal in both. As the results by Folkow *et al.* (1977) suggest largely equal glomerular filtration capacities in the NCR and SHR kidneys at this stage of life, the differences in deduced P_G increases shown in Fig. 4 right part, will fairly closely reflect differences in effective glomerular filtration pressure and filtrate volumes between SHR and NCR kidneys at given P_A levels. It should be noted how closely these curves of P_A and P_G resemble the $P_A - \text{GFR}$ curves shown in Fig. 1 of the paper by Folkow *et al.* (1977), as would be expected since they reflect largely the same processes, though measured and deduced in entirely different way.

Discussion

The present experiments illustrate the many difficulties involved when quantitative kinetic analyses of the renal vascular bed are performed. If, for example, the consequences of an altered vascular design are explored during maximal vasodilatation and constant perfusion, this also eliminates the active preglomerular autoregulation which normally keeps glomerular filtration pressure almost constant. It introduces, in return, just flow autoregulation, a consequence of the increases in renal tissue pressure (P_t) that follow upon enhanced glomerular filtration and tubular distension in connection with elevated



the relationship between P and GFR (ml/min/g dry weight) in artificially perfused isolated from 3-4 month old SHR and NCR, as earlier presented by Folkow *et al.* (1977), but here P increases according to Fig. 4. This serves to illustrate the relationship between the 'true' perfusion pressure for the renal vascular bed ($P_A - P_v$) and glomerular filtration rate in SHR and NCR kidneys. How this correction makes the curves much steeper though still with marked rightward shift. The curve in this way which can only be ascribed to structurally based elevation of the pre-post-glomerular resistance ratio.

on arterial pressure (P_A) (cf. Aukland 1976). Since the effective perfusion pressure for the renal vascular bed equals $P_A - P_v$, rather than P minus extra-renal P_v , the P increases are especially pronounced, and hence hemodynamically important, whenever glomerular filtration is much enhanced or/and tubular reabsorption depressed.

For such reasons comparative analyses of renal vascular resistances can be very misleading if based only on P_A and flow measurements, particularly if the kidneys differ also in their pre- and post-glomerular resistance ratio (R_{pre}/R_{post}), glomerular filtration capacity and tubular reabsorption. The influence of such factors on tubular pressure and hence on P can be fully appreciated concerning overall renal hemodynamics only if two different rates are used for the comparison, one which is filtered in the glomeruli and one which is able to pass across capillary walls, like the molecular oil kerosene (Wang 1948). With the type of perfusate the only possible contribution to 'passive autoregulation' via P would be if such marked intrarenal vascular distension occurs during P elevation that P is thereby increased significantly. This approach was utilized in the present comparison of the NCR and SHR renal vascular beds. It should be stressed that the same principal difficulties would be encountered if these problems were instead studied by micro-techniques, and the present 'whole organ' technique has here the great advantage of averaging pressures, flow and other events for the entire population of nephrons and their blood supply.

Fig. 3 makes it clear that the true vascular resistance to flow ($(P - P_v)/Q$) is moderately higher in SHR than in NCR of early adult age, while the reverse is true for the apparent resistance to flow ($(P_A - P_v)/Q$). In other words, the R increases with P elevations must be

much more pronounced in NCR than in SHR, which is also illustrated by direct P_{A} measurements shown in Fig. 2. These findings lend further support to earlier findings (Göthberg *et al* 1976 Folkow *et al* 1977) which strongly suggest the presence of a structurally based increase of $R_{\text{pre}}/R_{\text{post}}$ in early established SHR hypertension. The data further indicated close similarities between SHR and NCR concerning glomerular capacities and extent of tubular reabsorption in the prevailing experimental situation. Differences in these latter respects could otherwise have resulted in different P_{A} levels at P_{A} since they also affect the degree of tubular distension.

Based on the deduced $P_{\text{A}} - P_{\text{G}}$ relationships, shown in Fig. 4 it is possible also to make approximate corrections for the P_{G} changes occurring when P_{A} and GFR were varied in each other in adult SHR and NCR kidneys in the experiments by Folkow *et al* (1977). This seems justified as the same perfusate and perfusion technique was used in the two studies and the animals used were of the same age (3-4 months) and belonged to the same stock. By this P_{G} correction the more relevant relationship between the true pressure for renal perfusion ($P_{\text{A}} - P_{\text{G}}$) and GFR is obtained and these deduced relationships are illustrated in Fig. 5 for adult SHR and NCR. As would be expected, they show that GFR increases with arterial pressure rises when the true rather than the 'apparent' perfusion pressure is given along the abscissa, but still the SHR curve is almost parallelly placed some 25 mmHg to the right of the NCR one along the pressure rise.

This circumstance must imply that for equal arterial pressures the glomerular capillary pressure is 25-30 mmHg lower in adult SHR than in NCR, even when the renal vessels are completely relaxed. In the same situation the true total renal vascular resistance is 10 per cent higher in SHR to judge from Fig. 3. These data allow for approximate deduction of the structurally determined pre- and postglomerular resistance in SHR and NCR with respect to their relative differences. If in this situation total renal resistance is arbitrarily set to 100 and $R_{\text{pre}}/R_{\text{post}}$ to the relatively low level of 25/75 since the degree of active preglomerular autoregulation would reduce R_{pre} , the corresponding R_{pre} in SHR would be about 120 and 60/60 respectively at an MAP level of 100 mmHg for NCR. The only chance for the SHR kidney to maintain the same glomerular filtration pressure as the NCR one when perfused at 100 mmHg, would be to increase P_{A} up towards 150 mmHg for SHR which largely corresponds to the arterial pressure levels in the awake animal.

In other words, the renal barostat function is in SHR most efficiently 'reset' to a higher mean arterial pressure equilibrium by principally the same process of vascular adaptation which is responsible for structural autoregulation in other systemic circuits (Folkow *et al* 1974 Folkow 1975). It further seems as if the postglomerular resistance in the SHR kidney is, if anything, slightly reduced compared with NCR. This may not be surprising because with a high preglomerular resistance, which *in vivo* is reinforced by active autoregulatory and vasoconstrictor fibre influences, the mean transmural pressure along the postglomerular vascular sections may well be on average kept somewhat lowered in SHR compared with NCR despite the higher arterial pressure level in SHR. Since vascular design has been changed in the opposite direction if local distending pressure is kept subnormal (Folkow *et al* 1971 a), a modest reduction in the structurally set postglomerular resistance can then be expected contributing to the elevation of $R_{\text{pre}}/R_{\text{post}}$.

whether the structural increase of R_{pre}/R_{post} in SHR seems to be associated with a thickened media of preferentially the preglomerular vessels. This is suggested by the characteristic hyperactivity and increased maximal contractile strength shown by the SHR renal resistance vessels (Folkow *et al.* 1971 b), again a close analogue to the situation for the pre-arteriolar resistance vessels in other systemic circuits. It is known that the preglomerular resistance vessels are the major site for 'active' renal autoregulation and also for vasoconstrictor fibre adjustments. Such local and remote excitatory influences on the preglomerular resistance vessels would, as mentioned, tend to further accentuate the elevated R_{pre}/R_{post} in SHR. A structurally increased w/r in the preglomerular vessels implies a potentiation of local reduction even if the extent of smooth muscle activation is the same.

In the whole, the *per se* normal principle of rapid structural adaptation of heart and vessels to average changes in local load, shared by almost all tissues, has a series of most serious consequences for the induction and maintenance of hypertension. Besides causing early 'resetting' of systemic precapillary resistance (Folkow *et al.* 1973; Folkow 1975), the Frank-Starling relationship for the left ventricle (Hallböök 1975) and of the arterial baroreceptors (*cf.* Thorén 1978), such changes will in SHR primary hypertension efficiently reset also the long-term barostat function of the kidneys. This is evidently achieved by means of a structurally based elevation of the pre/postglomerular resistance in association with a preglomerular hyperactivity that is mainly a consequence of a structural w/r increase of these vessels, where the presence of a media thickening is well documented for the renal vascular bed in hypertension also in man (*e.g.* Furuyama 1962). To maintain glomerular filtration unchanged, mean arterial pressure must be kept correspondingly elevated in SHR and, should the filtration capacity later become reduced, arterial pressure must be even further elevated where perhaps a postglomerular constriction in such later stages may contribute.

The present experiments further illustrate how the hemodynamic techniques needed for quantitative analyses of the renal vascular bed also introduce often marked elevations of renal tissue pressure (P_t), and in a way which tends to mask the true vascular differences between SHR and NCR. This source of error was not fully realized in the initial study of renal hemodynamics in SHR (Folkow *et al.* 1971 b). Here SHR and NCR kidneys are perfused in parallel, with Tyrode solution containing as colloid 3 per cent coll, and the P_t increases must have affected mainly the estimations of total flow resistance by maximal dilatation, thus overestimating that in NCR and underestimating that in SHR. Such disturbances must even more seriously have affected the similar more recent findings of Colla and Vanhoose (1977) because the total lack of colloids in the perfusate used by these investigators must here have resulted in marked glomerular filtration even at low P_t values.

The approximate extent of P_t influences in these two *in vitro* studies may be illustrated by a following comparison to the normal *in vivo* situation. At 100 mmHg pressure level and during maximal vasodilatation, renal blood flow in normotensive rats is around 8 ml/g · min. Also here glomerular filtration increases during vasodilatation but the intact tubular reabsorption tends to keep the P_t increase relatively modest. Effective viscosity of blood at near normal hematocrit is about 3–2.5 cP at high flow rates (Djafarzadeh *et al.* 1970),

which would largely apply also for the kidneys. An extrapolation to 100 mmHg of the pressure-flow relationships for NCR kidneys during *in vitro* Ficoll-Tyrosine perfusion (Folkow *et al.* 1971 b) would here mean a flow around 8–10 ml/g min, but at a perfusate viscosity of only around 1–1.2 cP. In Collis and Vanhoutte's (1977) renal perfusion with Tyrosine solution with a perfusate viscosity about 0.7 cP flow was only some 5–6 ml/g min in the NCR as well as the SHR kidneys at a P_A level of 100 mmHg. In both these studies great care was taken to keep the renal vessels completely relaxed, but corrections for differences in perfusate viscosity reveal that the apparent renal resistance to flow (P/P_Q) was nevertheless about doubled in the study of Folkow *et al.* (1971 b) and increased four times in that of Collis and Vanhoutte (1977), compared with rat kidneys *in vivo*.

Clearly the P increases must have been formidable, particularly in the latter *in vivo* study where the lack of perfusate colloids eliminated all restrictions on glomerular filtration and hence on P elevations and probably also meant filtration across the tubular capillaries. In fact the true pressure head ($P_A - P_Q$) driving the renal perfusion in Collis and Vanhoutte's experiments at e.g. 100 mmHg, could only have amounted to some 40 per cent of the P when also renal vascular distensibility is taken into account as judged from the results in Fig. 3 left part. If then the R_{pre}/R_{post} is initially higher in the SHR kidneys, as seen above, P levels must have been correspondingly lower in the SHR kidneys compared with the NCR ones. In other words, the largely equal apparent vascular resistance (P/P_Q) in the SHR and NCR kidneys, observed by Collis and Vanhoutte, must in reality imply far lower true renal vascular resistance, $(P_A - P)/Q$, but where that in SHR is higher than in NCR largely in proportion to the difference in R_{pre}/R_{post} .

Obviously without knowledge of average P levels total renal vascular resistance cannot be properly compared in SHR and NCR in artificial perfusions, since the present structural R_{pre}/R_{post} increase in SHR inevitably leads to an underestimation of the vascular resistance, and largely in proportion to the increase of R_{pre}/R_{post} . Similar errors are involved in the study by Folkow *et al.* (1971 a), though being here more modest in part because P_A levels were kept lower partly because glomerular filtration was kept limited by the presence of perfusate colloid.

Moreover during tests for renal vascular smooth muscle constriction to nerve stimuli or to constrictor drug infusions, Collis and Vanhoutte used constant optimal perfusion which evidently corresponded to 4–5 ml/g min, where already a major P influence is present. Again, these pressor responses must have been markedly distorted by these high P levels, and to different extents in SHR and NCR. These high but different P levels would be further accentuated whenever vasoconstrictions during constant flow perfusion included also the postglomerular resistance vessels, as such constrictions further reduced filtration and hence P . At given active P_A elevations, the presence of an accentuated glomerular response in SHR would therefore always imply a relatively smaller P contribution to the P_A rise than in NCR.

Consequently the phenomenon of passive autoregulation via P changes will in the course of comparative analyses tend to greatly reduce the real differences between SHR and NCR concerning the extent of active true vascular responses, and then underestimate the responses more or less in proportion to the higher R_{pre}/R_{post} . These distortions must

been so marked in Colla and Vanhoutte's experiments as to largely mask even subtle differences between SHR and NCR kidneys with respect to R_{pre}/R_{post} and pre-renal vascular reactivity.

ahn, such sources of error would in principle be involved also in the vascular reactivity earlier performed on SHR and NCR kidneys by Folkow *et al* (1971 b), though being of much smaller impact. The reason is that flow and P_A were here kept much lower or 1/3, and the colloid content in the perfusate tended to restrict glomerular filtration area and hence also P increases. Thus, even though P also here tended to mask the differences in vascular reactivity between SHR and NCR, it was nevertheless evident from results that the SHR renal resistance vessels had a much higher maximal contractile strength and produced steeper resistance increases. Such a combination of vascular tests cannot be primarily ascribed to alterations in smooth muscle sensitivity or in nor-adrenergic characteristics, since they occurred also when the contractile proteins were fully engaged by barium ions, like calcium ions bypassing membrane events and the mechano-electrical coupling mechanisms. They can only be ascribed to the presence of an excess bulk of contractile tissue per unit vessel radius which, of course, by no means excludes concomitant deviations also in the functional characteristics of the vascular effector.

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References

- OLIN, K., Renal Blood Flow. *Internat. Rev. Physiol. Kidney and urinary tract physiology* 11, 1976, 11-23-79.
- AL, M. and P. M. V. VANDER, Vascular reactivity of isolated perfused kidneys from male and female spontaneously hypertensive rats. *Circulat. Res.* 1971 41 739-767.
- WOLFE, A. M., B. FOLKOW, B. ÖBERG and S. WILHE, A comparison of blood viscosity measured from *Acta physiol. scand.* 1970, 72, 70-84.
- AL, R., Vascular changes in hypertension—review and recent animal studies. In *Pathophysiology of hypertension of arterial hypertension*. Proc. of conference in Copenhagen 1975. Ed. G. Berglund, Almqvist and L. W. A. Lindgren and Söner AB, Mölndal, Sweden, 95-113.
- AL, R. and J. LARSEN, The interrelationship of some factors influencing renal blood flow autoregulation. *Acta physiol. scand.* 1964 87 163-176.
- AL, R. and B. LÖNN, The distensibility of the systemic resistance blood vessels. *Acta physiol. scand.* 1964 77 37-52.
- AL, R., O. GÖRANSSON, S. LARSEN and S.-E. RIKSTEN, Structural "resetting" of the renal vasculature in spontaneously hypertensive rats (SHR). *Acta physiol. scand.* 1977 102 270-272.
- AL, R., M. HALLBACK, Y. LINDGREN and L. WILHE, Renal vascular resistance in spontaneously hypertensive rats. *Acta physiol. scand.* 1971 b, 83 96-103.
- AL, R., HALLBACK, Y. LINDGREN, L. WILHE, I. ALBRECHT and S. J. LUTZ, Analysis of design and function of stress-coupled vascular sections in spontaneously hypertensive rats (SHR). *Acta physiol. scand.* 1974 90 654-656.
- AL, R., M. GILFILLAN, M. HALLBACK, Y. LINDGREN and L. WILHE, The hemodynamic consequences of arterial hypertension in spontaneously hypertensive and normotensive rats. *Acta physiol. scand.* 1974 90 332-341.

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Cardiac receptors with non-medullated vagal afferents in the rat

By

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Abstract

THORÉN, P., E. NORLÉN and S. E. RICKSTEN. *Cardiac receptors with non-medullated vagal afferents in the rat*. Acta physiol. scand. 1979 105: 295-303.

Characteristics of 22 atrial receptors in 15 normotensive adult male Wistar rats were investigated. All vagal afferents were included in the C-fibre group with conduction velocities from 0.4 to 1.2 m/sec. Atrial medullated receptors or ventricular C-fibre endings were found. Two receptors were located in the atrium and 2 receptors throughout left atrium. Upon elevation of the left atrial pressure the receptor type was markedly elevated with thresholds from 2.5 to 9 mmHg in mean left atrial pressure. Ten of these receptors displayed clear cardiac rhythmicity upon activation and the discharge correlated with heart rate, indicating distension as the cause of receptor activation. The maximal firing rate in most receptors was very high (up to 70 Hz), but 8 receptors had maximal firing rates below 25 Hz. These low frequency receptors had higher threshold and showed more irregular firing. Thus, there is a substantial class of atrial receptors with vagal non-medullated afferents in the rat heart and the thresholds for many of these receptors are so low that they are likely to be active during normal conditions.

Evidence of receptors in the heart was first suggested more than 100 years ago by von Kries and Hart (1867), but it is first when neurophysiological techniques became available that these receptors could be studied in detail (Jarisch and Zotterman 1948; Zotterman 1951). Thus, in hearts of cats and dogs there exists a population of receptors with medullated afferents mainly located at the vein-atrial junctions. Some of these receptors respond (Paloutsek 1951) to the atrial contraction (A-receptors) and other respond to the atrial filling (B-receptors).

There also exist a large number of receptors with non-medullated afferents in the heart (Jarisch and Zotterman 1948; Coleridge *et al.* 1964; Sleight and Widdicombe 1965; Öberg 1971; Thorén 1972; Thorén 1976, 1977), but these receptors seem to be located throughout the entire heart and they respond mainly to changes in atrial filling (Thorén 1976, 1977).

The aim of the present study was to identify different types of vagal sensory endings in the heart of the rat and to describe their characteristics in relation to changes in cardiac function. Interestingly enough we found a large population of C-fibre endings in the atria, no medullated atrial afferents and no ventricular afferents.

- FOLKOW, B., M. HALLBÄCK, Y. LUNDGREN, R. SIVERTSSON and L. WEISS, Importance of adaptive changes in vascular design for establishment of primary hypertension, studied in man and in spontaneously hypertensive rats. *Circulat. Res.* 1973 Suppl. 1 32 and 33, 2-16.
- FURUYAMA, M. Histometrical investigations of arteries in reference to arterial hypertension. *J. Exp. Med.* 196... 76 388-414.
- GÖTHBERG, G., M. HALLBÄCK, S. LUNDIN, S. E. RICKSTEN and B. FOLKOW. A comparison of total peripheral resistance in normotensive control rats and spontaneously hypertensive rats (SHR). *Clin. exp. Res. Physiol.* 1976. Suppl. 3 79-82.
- GUYTON, A. C., T. B. COLEMAN, A. W. COWLEY Jr., R. D. MANNING Jr., R. A. NORMAN and J. D. GIBSON. A systems analysis approach to understanding long-range arterial blood pressure control in hypertension. *Circulat. Res.* 1974. 35 159-176.
- HALLBÄCK, M., B. ISAKSSON and E. NORESSON, Consequences of myocardial structural adaptation on left ventricular compliance and the Frank-Starling relationship in spontaneously hypertensive rats. *Acta physiol. scand.* 1975 94 259-270.
- HALLBÄCK, M., Y. LUNDGREN and L. WEISS, The distensibility of the resistance vessels in spontaneously hypertensive rats (SHR) as compared with normotensive control rats (NCR). *Acta physiol. scand.* 1976 90 57-68.
- NORESSON, E., M. HALLBÄCK and Å. HJALMARSSON, Structural "resetting" of the coronary vascular system in spontaneously hypertensive rats. *Acta physiol. scand.* 1977 101 363-365.
- THOMAS, P., Role of cardiac vagal C-fibres in cardiovascular control. *Rev. Physiol. Pharmacol. and Ther.* 1978. In press.
- WAUGH, W. H., Flow as a function of arterial pressure in the oil perfused kidneys. *Circulat. Res.* 1966 6, 107-115.

Cardiac receptors with non-medullated vagal afferents in the rat

By

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Abstract

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Characteristics of 22 atrial receptors in 15 normotensive adult male Wistar rats were investigated. All receptor afferents were included in the C-fibre group with conduction velocities from 0.4 to 1.2 m/sec. Atrial medullated receptors or ventricular C-fibre endings were found. Two receptors were located in atrium and 2 receptors throughout left atrium. Upon elevation of the left atrial pressure the receptor large as markedly elevated with thresholds from 2.5 to 9 mmHg in mean left atrial pressure. Ten of atrial receptors displayed clear cardiac rhythmicity upon activation and the discharge correlated with waves, indicating dominance in the course of receptor activation. The maximal firing rate in most receptors was high (up to 70 Hz), but 8 receptors had maximal firing rates below 25 Hz. These low frequency receptors had higher threshold and showed more irregular firing. Thus, there is substantial relation of atrial receptors with mixed non-medullated afferents in the rat heart and the thresholds for many of these receptors are so low that they are likely to be active during normal conditions.

The existence of receptors in the heart was first suggested more than 100 years ago by von Kries and Hirt (1867), but it is first when neurophysiological techniques became available if these receptors could be studied in detail (Jarisch and Zotterman 1948, Zotterman 1951). Then, in hearts of cats and dogs there exists a population of receptors with medullated afferents mainly located at the vein-atrial junctions. Some of these receptors respond (Palmiter 1973) to the atrial contraction (A-receptors) and other respond to the atrial filling (B-receptors).

There also exist a large number of receptors with non-medullated afferents in the heart (Jarisch and Zotterman 1948, Coleridge *et al.* 1964, Sleight and Widdicombe 1965, Öberg and Thorén 1972, Thorén 1976, 1977), but these receptors seem to be located throughout the entire heart and they respond mainly to changes in atrial filling (Thorén 1976, 1977).

The aim of the present study was to identify different types of vagal sensory endings in the heart of the rat and to describe their characteristics in relation to changes in cardiac pressure. Interestingly enough we found a large population of C-fibre endings in the atria, but no medullated atrial afferents and no ventricular afferents.

Methods

Experiments were performed on 15 normotensive adult male Wistar rats (weighing 320–350 g, up to 7 weeks). In some animals the awake mean arterial pressure (MAP) was recorded via a PE 50 catheter inserted into the tail artery under light ether anaesthesia. The animals were then anaesthetized with pentobarbital (Nembutal® 40–50 mg/kg i.v.) i.a. or i.p. The anaesthesia was maintained by 10–15% pentobarbital in repeated injections i.a. The trachea was cannulated with a PE 200 tube and positive-pressure ventilation with pure oxygen was induced with a frequency of 28 per minute and end-expiratory pressure of 12 to 15 cm water. In preliminary experiments (2 rats) this type of artificial ventilation gave similar values for arterial PO_2 (350–450 mmHg) and PCO_2 (35–45 mmHg). To prevent acidosis small doses of $NaHCO_3$ (0.1–0.2 mM) were injected at intervals. Muscle movements were prevented by gallamine F dil® 4–5 mg/kg b.w. in repeated i.v. injections. Rectal temperature was measured electronically and maintained at 37–38°C by means of a thermostatically controlled shielded heat pad.

Surgical procedures

The vagus nerve and the carotid artery were dissected free in the neck either on the right (12 rats) or left (3 rats). The thorax was opened via a bilateral, intercostal, trans-sternal incision. The pericardium was split and snares were placed around the ascending aorta and the inferior caval vein near the heart.

A thin catheter (PE 10) was placed with its tip in the left atrium via the right middle long vein and connected to an ultra low displacement transducer (Gaeltec 3 EA). The right upper and middle long veins were then removed. The arterial blood pressure was recorded in the tail artery. A jugular vein was cannulated. The vagal trunk was identified just above the lung root and cut in order to eliminate afferent activity from the lungs and abdominal viscera.

Recordings

The right or left vagus were cut centrally and placed on a dissection plate. The plate was situated in a constructed box by the surrounding muscles and skin and filled with body warm mineral oil. The nerve was carefully dissected away by means of sharp forceps under a binocular Zeiss dissection microscope. Thin filaments were then obtained and placed on a thin bipolar silver electrode and connected to a 6-lead probe (HIP 511E) and the nerve spikes were amplified (Gram amplifier P511).

The high frequency cut off was normally set at 1000 Hz and the low frequency cut off at 30–50 Hz. The output from the amplifier was displayed both on an oscilloscope (Tektronix 511S) and on a UV recorder (ABEM 3651) writing intermittently with fast speed (10–200 cm/s). The amplifier was also connected to a loudspeaker and to a discriminator equipped with a rate meter which could count nerve spikes exceeding a preset value or only spikes with amplitudes between two determined levels. The output of the rate meter, the arterial blood pressure and the mean left atrial pressure were recorded on a 4-lead Grass polygraph (mod. 7). The ECG, the arterial and the left atrial pressure waves were also recorded on the UV recorder together with the nerve activity.

Experimental procedure

As a screening procedure to find receptors in the left side of the heart, the activity in all dissected filaments was observed during shortlasting (3–6 s) occlusions of ascending aorta. Every filament that responded to this manoeuvre was dissected further until a filament with only one active site was found. The receptors were localized by probing the heart with a thin probe in most instances after the animal had opened its heart. The total conduction time was obtained by placing a stimulation electrode connected to a Grass (S4) stimulator over the receptor site and recording the evoked potential in the filament in the neck. The conduction velocity was estimated from the approximated length of conduction pathway and the total conduction time.

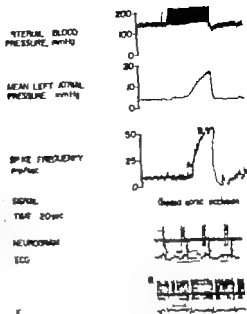
Receptor activity was recorded during control conditions and during graded elevation of left atrial pressure induced by graded aortic occlusion. The receptor response to electrically induced ventricular fibrillation of the ventricles was observed in some animals.

The significance of changes was determined by Student's *t* test and values are presented as mean \pm S.E.

Results

This study is based on recordings from 22 receptors in 15 normotensive rats. 4 of the receptors were dissected from the left cervical vagus and 18 receptors from the right cervical vagus.

Effects of progressive obstruction of the aorta on aortic blood pressure, mean arterial pressure and spike frequency in left atrial C-fibers. The aortic blood pressure increased just below the aortic valve or just in the right carotid artery. The latter spike frequency recordings corresponds to program below. Initially the receptor has a low modulated rhythm with 1-2 spikes/s. As resistance increases in left atrial pressure (mean A) the receptor is firing with 1-2 spikes each wave. Upon further atrial distension this started increase in receptor firing up to 16 spikes per cardiac cycle.



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total conduction time from the receptor site to the recording electrode was from 125 ms and the conduction velocities were from 0.4 to 1.2 m/s (mean \pm S.E., 0.7 ± 0.1).

Location

The receptors were located in the heart by probing with a fine plastic probe. Two right atrial receptors were located, one to the superior caval vein and the other to the base of the atrial appendix. The characteristics of the right atrial receptors were not studied in this experiment because the right atrial pressure was not measured. They however behaved similar to left atrial endings with a clear cardiac rhythmicity upon activation during pulmonary artery occlusion. The left atrial receptors were located throughout the entire left atrium, in the pulmonary vein, left atrial junctions and in the left atrial appendix. 10 of the receptors were located in the opened non-beating heart and the other 12 in the intact heart. Of these 22 receptors 5 were located in the pulmonary veins close to the atrium and 4 were located in the appendix.

Response to increased atrial pressure

The receptors responded to increased pressure in the atria during a brief occlusion of the ascending aorta with maximal frequencies from 1 to 70 Hz (35 ± 4). The response of the receptors were also examined during ventricular fibrillation. Then there was a clear rise in atrial pressure and concomitant increased receptor firing as observed in left atrial C-fibers in the cat (Thoren 1976).

Methods

Experiments were performed on 15 normotensive adult male Wistar rats (weighing 320–500 g, up to 7 weeks). In some animals the awake mean arterial pressure (MAP) was recorded via a PE 38 catheter inserted into the tail artery under light ether anaesthesia. The animals were then anaesthetized with pentobarbital (Nembutal® 40–50 mg/kg b.w.) i.a. or i.p. The anaesthesia was maintained by 20–30 mg pentobarbital in repeated injections i.a. The trachea was cannulated with a PE 200 tube and positive pressure ventilation with pure oxygen was induced with a frequency of 120 per minute and end-expiratory pressure of 12 to 15 cm water. In preliminary experiments (2 rats) this type of artificial ventilation values for arterial PO₂ (350–450 mmHg) and PCO₂ (35–45 mmHg). To prevent acidosis small amounts of NaHCO₃ (0.1–0.2 mEq) were injected at intervals. Muscle movements were prevented by gallium sulphide (4–5 mg/kg b.w.) in repeated i.v. injections. Rectal temperature was measured electronically and maintained at 37–38°C by means of a thermostatically controlled shielded heat pad.

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Experimental procedures

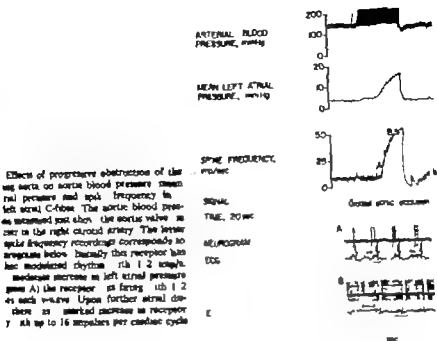
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Effects of progressive obstruction of the aortic arch on aortic blood pressure, mean left atrial pressure and spike frequency in left atrial C-fibres. The aortic blood pressure measured just above the aortic valve is shown in the right carotid artery. The latter spike frequency recordings corresponds to a pressure below, because this receptor has been modelized rhythmically with 1.2 impulses/sec. moderate increase in left atrial pressure (see A) the receptor is firing with 1.2 impulses/sec. Upon further atrial distension a marked increase in receptor firing up to 16 impulses per cardiac cycle

The total conduction time from the receptor site to the recording electrode was from 1.5 ms and the conduction velocities were from 0.4 to 1.2 m/s (mean \pm S.E., 0.7 ± 0.1 m/s).

Location

The receptors were located in the heart by probing with a fine plastic probe. Two right atrial receptors were located, one to the superior caval vein and the other to the base of the atrial appendix. The characteristics of the right atrial receptors were not studied in detail because the right atrial pressure was not measured. They however behaved similarly to the left atrial endings with a clear cardiac rhythmicity upon activation during pulmonary artery occlusion. The left atrial receptors were located throughout the entire left atrium, in the pulmonary veins, left atrial junctions and in the left atrial appendix. 10 of the receptors were located in the opened non-beating heart and the other 12 in the intact heart. Of these 22 receptors 5 were located in the pulmonary veins close to the atrium and 4 were located in the appendix.

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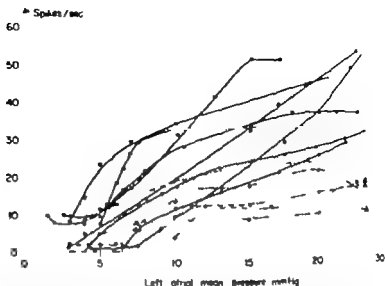


Fig. 2. The pressure-response curves for 13 different left atrial receptors in 12 rats. The interrupted line indicates receptors of the low frequency type with maximal firing rates less than 25 Hz and the other receptors belong to the group of high frequency receptors. The sign Δ indicates receptors with an irregular charge pattern upon activation.

The response to a graded increase in left atrial pressure was also examined in 13 left atrial C fiber endings. The typical response is shown in Fig. 1. This receptor had a spontaneous activity of 1 or 2 impulses per cycle and after correction for the total conduction time (indicated by the asterisks) the receptor was activated during the v wave. Upon aortic occlusion there was a marked increase in left atrial pressure and a concomitant increase in firing rate with a maximal activity of 55 Hz or 16 impulses per cardiac cycle. The thresholds for activation for all the left atrial receptors ranged from 2.5 to 5 mmHg in mean left atrial pressure.

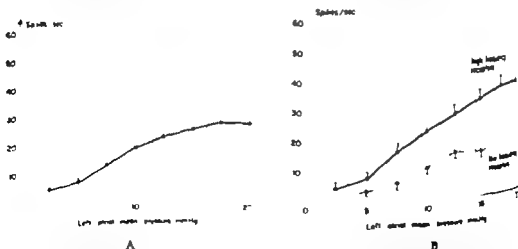
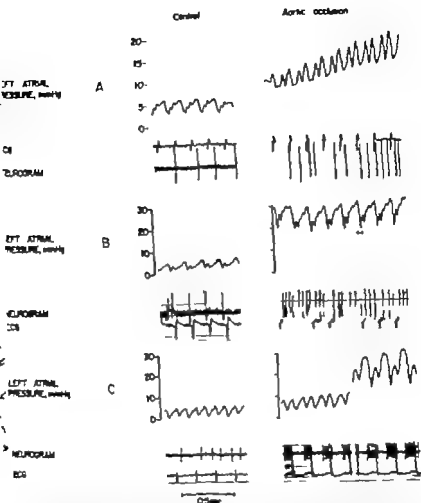


Fig. 3 A. Activity in non-medullated vagal afferents from the left atrium during increases in left atrial pressure caused by graded aortic occlusion (mean \pm S.E. of recordings from 13 single fibres).

Fig. 3 B. Relationship between the mean left atrial pressure and firing frequency in 8 high frequency left atrial receptors and 5 low frequency left atrial receptors upon graded aortic occlusion (mean \pm S.E.).



1 Left atrial pressure, ECG and activity in 3 left atrial C-fibre endings in 3 rats. The receptor in panel A shows a low frequency irregular discharge upon activation and is included in the low frequency type of receptor. The receptor in panel B is the only low-frequency receptor, showing cardiac rhythmicity characteristic of left atrial pressure. The receptor in panel C is in the control situation activated alternately by the a- and v-wave, but upon elevation of the left atrial pressure the receptor shows a progressive type in relation to the v-wave. (The asterisks indicate the corrected position in the cardiac cycle of the low activation.)

average, 1.4 ± 0.5 mmHg). The pressure-response curves for the individual receptors are shown in Fig. 2. Some receptors showed very high firing frequencies up to 35 Hz and some others fired with rather low frequencies. Fig. 3 A shows the mean activity in this group of receptors in relation to left atrial pressure and is based on the data shown in Fig. 2.

High and low frequency receptors

A surprising finding in this study was the marked variation in the pressure-response curves. Some endings showed a regular discharge with very high maximal frequencies. These high

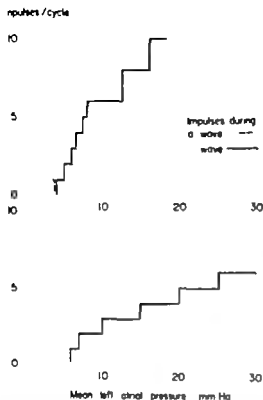


Fig. 5. Number of impulses per cardiac cycle in relation to the mean left atrial pressure for 2 different left atrial C-fibre endings. Impulses generated during the P-wave are plotted separately from those generated during the V-wave. At low pressure receptors show a low frequency a-wave discharge upon elevation of the left atrial pressure but no V-wave activity. At higher pressures the V-wave receptors were markedly activated during the V-wave.

frequency regular discharging receptors behaved very much as the modulated atrial receptors found in the cat (Paintal 1973). Other atrial C-fibre endings had similar characteristics to the earlier described atrial C fibres in the cat with a fairly low frequency activity. A attempt was therefore made to analyse the characteristics of high and low frequency receptors separately. The receptors with maximal activity above 25 Hz were called high frequency receptors and receptors with maximal frequencies below 25 Hz were called low frequency receptors. In Fig. 2 the high frequency receptors are indicated by solid line and the low frequency receptors with an interrupted line. Fig. 3 B shows the mean pressure-response curves for low and high frequency receptors separately.

Is there then any evidence that these arbitrary grouping of the atrial C-fibre receptors really could represent 2 functionally different groups of receptors? Some evidence indicate that. First the threshold for the high frequency and low frequency receptors were 4.6 ± 0.6 mmHg respectively 6.9 ± 0.7 mmHg ($p < 0.05$). Further all the high frequency receptors displayed a clear cardiac rhythmicity upon activation but 4 of the 5 low frequency receptors (as indicated in Fig. 2) displayed mainly irregular activity upon activation.

The firing pattern in the cardiac cycle

The firing pattern in the cardiac cycle was established in 16 receptors because the conduction time from the receptor site to the recording electrode was measured. Upon activation 4 receptors showed an irregular discharge with no obvious relation to the cardiac cycle as indicated in Fig. 2. These receptors were of the low frequency type. All the other receptors showed a cardiac modulated rhythm upon activation with up to 16 impulses per cardiac cycle. The firing was mainly correlated to the V-wave of the cardiac cycle. The low frequency receptors showed a low frequency activity upon activation.

had 1-3 impulses during each a-wave. Two receptors showed both v- and a-wave responses, with 1-2 impulses per a-wave at high atrial pressures. The typical discharge in ventral atrial C-fibre endings is showed in Fig. 4. Panel A shows a low frequency receptor and this ending displays irregular discharge during the control situation and during occlusion. Panel B shows the only low frequency receptor which displayed an evident rhythmicity (indicated by the asterisks). Panel C shows a typical high frequency receptor. This receptor shows in the control situation both an a- and v-wave discharge. When the left atrial pressure is moderately increased the receptors discharged with 4-5 impulses each v-wave and upon further increase in left atrial pressure the receptor discharge up to 10 impulses each v-wave.

Fig. 5 the number of impulses per cardiac cycle is plotted against the mean left atrial pressure. The receptor in the top panel is at low pressures activated with 1 impulse each v-wave. Upon pressure elevations the receptor discharge with up to 10 impulses per cardiac cycle. The receptor in the lower panel is only activated during the v-wave.

Discussion

The existence of atrial receptors with vagal modulated afferents has been known since many years (cf. Parnall 1973). These receptors have two different types of firing. One type of receptors is discharging mainly during the v-wave and these receptors are called B-receptors. The other type is discharging during the a-wave and these receptors are called A-receptors. However, in the *in vitro* situation these two types of receptors have similar basic properties (Hägg *et al.* 1974). There is also a substantial population of endings with non-modulated afferents in the heart. The main receptor station in cats and dogs seems to be the left atrium (Jarisch and Zotterman 1948, Coleridge *et al.* 1964, Sleight and Widdicombe 1965, Thoren 1977) but there is also a lot of atrial C-fibre endings in the atria (Coleridge *et al.* 1976, Thoren 1976). These atrial C-fibres in the cat respond mainly to increased atrial filling and some receptors also could respond to the atrial contraction (Thoren 1976). The thresholds for these receptors were somewhat higher and the firing frequencies were lower for the modulated receptors in the atria (Thoren 1976, Thoren *et al.* 1976). In this study the characteristics of atrial receptors in the rat are described for the first time. Earlier studies Kottsson and Sjöstrand (1974) have examined the characteristics of intracardiac afferents dissected from branches of the superior laryngeal nerve in rats. However, these authors never localized the receptors adequately and other studies indicate that the thoracic afferents in the superior laryngeal branches of the rat are not coming from the heart but from the aortic arch (Andrew 1954).

Receptor characteristics

In cats and dogs, atrial receptors in the rat respond mainly to atrial distension and are activated by low or moderate elevations in atrial pressure with thresholds from 2.5 to 9 mmHg. Thus, a substantial population of the receptors are active at normal left atrial pressure, which is about 5 mmHg (Noren *et al.* 1978). Upon activation most of the receptors discharge with a clear cardiac rhythmicity during the v-wave indicating atrial distension as a main mechanism of receptor activation.

High and low frequency receptors

There is a marked variation in the receptor characteristics in the individual receptor. Some receptors had low thresholds (2.5–5 mmHg) and respond upon atrial distension with high firing rates up to 60 Hz and with cardiac rhythmicity. Other receptors had higher thresholds (5–9 mmHg) and responded with low frequency (less than 25 Hz) discharge upon activation. The high frequency type of receptor seemed to have properties to the medullated fibre group in the cat. The thresholds for this high frequency receptor with cardiac rhythmicity were significantly lower than for the receptors with frequent and irregular activity.

An interesting question is then whether the high and low frequency receptors are in the same receptor system or constitute two different types of endings. As discussed in some evidences are speaking in favour of the latter concept. Thus, the low frequency receptors had significantly higher thresholds and discharged mainly (80%) irregularly on activation, indicating that they might constitute a different population of receptors. Interestingly enough, the aortic nerves of the rat also contains two types of afferent baroreceptor C-fibres. One type is discharging with high frequent regular activity and the other is discharging with low frequencies and with a more irregular type of activity (Thoren 1977). It might thus be a general phenomenon in the rat that the afferent information from baroreceptor and cardiac receptors is transmitted mainly in non-medullated fibres. One reason might simply be that these animals are small enough to allow for a time delay in afferent C-fibres. Interestingly enough the cervical vagus of the mice consists of about 8 000 fibres of which the non-medullated fibres constitute 92% (Inoue 1975). This fact will also support the concept that the afferent information in small rodents is mainly transmitted in C fibres.

Function of atrial receptors

In cats and dogs the function of the atrial medullated fibres seems to be different from the function of the atrial C-fibres (Thoren *et al.* 1976). It is thus of interest whether atrial low frequency receptors in the rat have different function from the atrial low frequency receptors. However, recently the effect of elevation of left atrial pressure on the reflex inhibition of renal sympathetic nerve traffic was examined in anaesthetized baroreceptor denervated rats (Ricksten *et al.* 1978). Upon plasma volume expansion there was a marked inhibition of renal nerve traffic due to activation of receptors in the left side of the heart and the threshold for the inhibition was about 4–5 mmHg which is in the same range as the thresholds of the high frequency receptor in this study. Thus, there is no evidence that the high frequency C-fibres as recorded in this study should not induce a vasomotor inhibition in the same way as the atrial C-fibres in the cat (Thoren *et al.* 1976).

Absence of ventricular receptors in rats

Another peculiar finding in this study was that we were not able to localize any left ventricular receptors with non medullated afferents. In the cat (Thoren 1977) and the dog (Merridge 1964; Sleight and Widdicombe 1965) the left ventricular C-fibre endings consist of a large number of fibres and the left ventricle seems to be the major receptor station for afferent C-fibres in the heart. There are at least two different explanations why we were

and any left ventricular C-fibre endings in the rat. First, it is possible that the number of endings in the heart is so low or that the thresholds of these C-fibres are so high that they are difficult to find. Indeed, in two instances we recorded from a C-fibre located in the heart which was responding to aortic occlusion with a low frequency, irregular discharge pattern and these receptors were markedly activated upon probing over the ventricular surface. However the definite localization of these receptors could not be achieved in the opened nonbeating heart and thus the precise localization could not be achieved. Another explanation is that the cutting of the vagal branches just above the heart might eliminate ventricular afferents but not the atrial afferents. However there is reason to believe that atrial and ventricular afferents should have a different afferent pattern in the rat.

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References

- B. L. A baroreceptor pathway for aortic baroreceptor impulses. *J. Physiol. (Lond.)* 1954, 125, 353-362.
- J. O. P. BRAMMING, K. HINDOY and M. RÖRSTEDT. The afferent discharge pattern of atrial mechanoreceptors in the cat during axonal stretch of atrial strips *in vitro*. *J. Physiol. (Lond.)* 1974, 232, 1-32.
- A. VOY and L. HUNT. Über die physiologischen Wirkungen des extrinsischen Vagusnervens. *Unters. Physiol. Wittenberg* 1867, 1, 75-156.
- ROD, R. M., J. C. G. COLCLOUGH and C. KATO. Cardio receptors in the dog, with particular reference to types of afferent ending in the ventricular wall. *J. Physiol. (Lond.)* 1964, 174, 323-339.
- ROD, R. M., J. C. G. COLCLOUGH, A. DAWSON, C. KATO, J. C. LUCK and P. SIMON. Impulses in conducting vagal fibres from afferent endings in the ventricle, atria and arteries of dogs and cats. *Circ. Res.* 1973, 33, 87-97.
- K. Electro microscopic analysis of the vagus nerve in the cat, its fiber composition, degeneration relationship with the superior cervical ganglion. *J. Amer. Soc. 1973*, 50, 1-14.
- U. A. and Y. ZOTTERMAN. Depressor reflexes from the heart. *Acta physiol. scand.* 1948, 16, 31-51.
- VOY, E. and T. SÖDERLUND. Impulse discharges from the low pressure side varying with the central volume. *Physiol. Arch. ges. Physiol.* 1974, 325, 105-114.
- VOY, E., S.-E. RASTENFELT and P. THOREN. Left atrial pressure in normotensive and spontaneously hypertensive rats. Submitted to *Acta physiol. scand.*
- VOY, E. and P. THOREN. Studies on left ventricular receptors, signalling in non-medullated vagal afferents. *Acta physiol. scand.* 1972, 87, 145-165.
- U. A. S. Vagal sensory receptors and their reflex effects. *Physiol. Rev.* 1973, 53, 159-222.
- VOY, E., S.-E. RASTENFELT and P. THOREN. Inhibition of vagal sympathetic nerve traffic from cardiac receptors in normotensive and spontaneously hypertensive rats. *Acta physiol. scand.* 1979, 1, preprint.
- VOY, E. and J. G. WIDJONOWSKI. Action potentials in fibres from receptors in the epicardium and myocardium of the dog left ventricle. *J. Physiol. (Lond.)* 1963, 181, 235-258.
- VOY, E. Atrial receptors with non-medullated vagal afferents in the cat. Discharge frequency and its relation to atrial pressure. *Circulation Res.* 1978, 39, 357-362.
- VOY, E. Characteristics of left ventricular receptors with non-medullated vagal afferents. *Circulation Res.* 1979, 40, 415-421.
- VOY, E., D. E. DONALD and J. T. SMITH. Role of heart and lung receptors with non-medullated vagal afferents in circulatory control. *Circulation Res.* 1976, 38, Suppl. 11, 2-9.
- VOY, E., W. R. SAUM and A. M. BROWN. Characteristics of aortic baroreceptors with non-medullated afferents in the rat. *Circulation Res.* 1977, 40, 321-337.
- VOY, E. and Y. ZOTTERMAN. Impulses from hjärta. *Nordiskt Medicin* 1948, 38, 1182. (Webbtryck utgivet av förlaget)

Hepatic portal vein infusion of glucose on sodium excretion in the rat

By

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Abstract

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Anesthetized rats were prepared with catheters in the hepatic portal (HPV) and femoral (FEM) veins, the bladder or ureters. In some experiments the left kidney was denervated. HPV infusion of 1 M glucose at 2 ml/h for 20 min increased Na excretion by the kidney when given as a second infusion. Bilateral vagotomy eliminated the increase in Na excretion during glucose infusion and uncovered a delay in Na and K excretion. Renal denervation attenuated the increase in Na excretion to HPV glucose. Infusion of glucose had variable effects, increasing Na excretion only after vagotomy. The results are interpreted to suggest that central and vagal receptors tend to enhance Na excretion whereas afferent nerve efferents and humoral mediator(s) have the opposite effect.

Key words: glucoreceptors, hepatic portal vein, Na excretion, vagal afferent nerves, K excretion.

Neurophysiological (Schmitt 1973; Nijima 1969) and behavioral (Blake and Lin 1978; Novin, Sanderson and VanderWeele 1974) evidence exists for the presence of glucose-sensitive receptors in the portal circulation to the liver. The vagus apparently carries inhibitory information and the splanchnic nerves excitatory although both effects depend on experimental conditions including time of day (Schmitt 1973; Novin *et al.* 1974). Most of the work done has been related to food intake but hepatic portal infusion of 2 M glucose has been shown to stimulate saline drinking and suppress water drinking in rats (Blake and Lin 1978). This effect was not reproduced by femoral vein infusion of glucose or by hepatic portal vein infusion of equimolar sucrose or fructose (Blake and Lin 1978) thereby differentiating the system from that involved in suppressing food intake (Booth and Booth 1976).

We know of no studies on renal function during infusion of hypertonic glucose into the hepatic portal vein. Hypotonic (2.5%) glucose had no effect on Na excretion during femoral vein infusion (Daly, Roe and Horrocks 1967) and similar negative results have been reported for infusions of hypertonic sucrose (Passo, Thornborough and Rothblat 1976).

of the above, we deemed it appropriate to examine the effect of hepatic portal infusion of hypertonic glucose on this other aspect of the salt and water balance system, i.e. excretion of Na and water by the kidneys.

Methods

Experiments were done on male Sprague-Dawley rats weighing between 250 and 350 g. All rats were used with loactin (Chase Fabre Promonta, Elmbl, Hainburg, W. Germany), 120 mg/kg p. and fed at body temperature of about 37°C with servo-controlled heating pad. The trachea, one artery and both femoral veins are exposed and cannulated with polyethylene tubing. The abdomen opened by median incision and, in all but two experiments, the appendix gently retracted to exposing mesenteric veins. One is cannulated through small needle hole and the tubing fixed by suturing to the mesentery. In two experiments only femoral vein infusions were made. Urine bladder is cannulated directly for collection of urine, in others both urters were cannulated and urine from both kidneys collected in the same tube or if the left kidney was deservated, in separate deservation was accomplished by stripping the pedicle of all visible nerv. fibers (under dissecting opt) and applying crystal of phenol. (It was not crucial to the purpose of the experiment to assure no deservation by transplantation.)

Arterial blood pressure was recorded from the femoral artery using Statham transducer and ink recorder. No blood samples were drawn. Urine volume was estimated by re-weighing of pre-weighed on tubes, analyses for Na and K concentration in urine samples were by atomic absorption spectroscopy. Urine samples were tested for the presence of glucose during periods of glucose infusion. Statistical protocols are similar except for the order of glucose infusions, i.e. whether into hepatic or femoral vein first or second and whether or not vagotomy was performed. In the first 5 experiments were given 3 to 5 ml of 0.85% NaCl solution i.v. over 5 min and then saline was infused into hepatic portal and femoral veins at 2 ml/h. In the remaining experiments, the rats received 1 ml of 5% glucose over 3 min period and were then given the minimal solution into the femoral vein at 2 ml/h and saline solution into the hepatic portal vein at 2 ml/h. One half (early experiments) to one hour after start of the infusions and completion of the operative preparation, the collection of 10 min urine was begun. After 10 to four "control" samples had been collected, 1 M glucose 2 ml/h was infused into femoral vein or the hepatic portal vein for 20 min. This infusion is divided, i.e. if infused into hepatic portal vein, the saline being infused there was shifted to the second femoral vein. (In the experiments with only femoral vein infusion, the 1 M glucose infusion was subdivided for the 0.85% NaCl and was not additive.) After from three to five "recovery" periods (30 to 30 min) the 1 M glucose was repeated into the same or the other vein. In some experiments, as many as 4 glucose infusions were in some experiments, bilateral cervical vagotomy (requiring 3 ml 5 min) was carried out after two glucose infusions and then one or two more repeated. The numbers of infusions for each vein whether given as the first infusion of glucose or second (see more) are indicated with the results.

Estimation of percent change in urine flow, Na or K excretion was done by linear interpolation between the immediately preceding control value and either the first or the third recovery period following infusion as described with the results. These approaches were used because a truly steady state was achieved; urine flow was sometimes increasing and sometimes decreasing. On occasion of occurrence, an infusion was given just prior to peaking or troughing of an oscillation in excretion. Such infusions included because of the unsuitability of using linear interpolation to predict values between statistical error between means as by "t" test for unpaired values as well as by paired comparisons for linear and deservated kidneys.

Results

Averaged values for urine flow, Na, and K excretion are shown for the studies on hepatic portal vein (HPV) infusion of glucose in Fig. 1. Two control, two infusion, and three recovery periods (all 10 min duration) are shown for each type of infusion. First infusion was that glucose was infused into HPV before being given elsewhere and second infusion was that glucose had already been given into either HPV or the femoral vein (FEM).

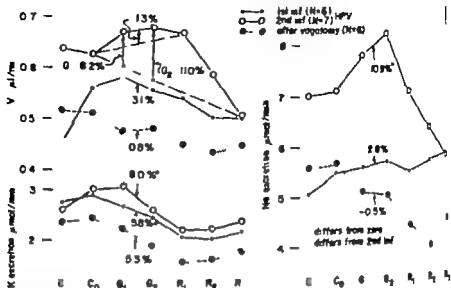
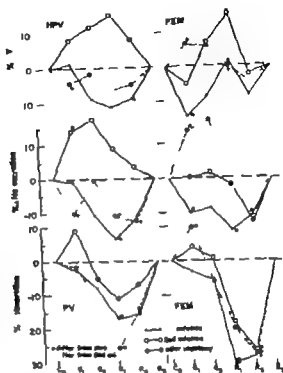


Fig. 1 Urine flow (V), Na and K excretion before (C and C_0), during (G_1 and G_2), and after (R_1 and R_2) HPV infusion of glucose. For explanation of 1st and 2nd infusion and for calculation of percent change see text.

All but one of the post vagotomy infusions were second infusion or more. (There were differences between second or third infusions.) The two methods used to calculate percent change in function are illustrated in the upper left part of Fig. 1 on the values for urine flow (V). In the first, interpolation was between the control period just preceding glucose infusion (C_0) and the first recovery period (R_1). The percent change was calculated from the difference between actual and predicted means for the average of G_1 and G_2 . Mean changes using this method are included in Fig. 1. In the second approach, interpolation was between C_0 and R and percent change from predicted was calculated for each of the intervening periods, namely G_1 , G_2 , R_1 and R_2 . The latter approach is believed to be more acceptable. Na and K excretion seemed to be altered following glucose infusion. For example, a decrease in K excretion following glucose infusion would seem more likely than the increase calculated by the C_0 to R method.

The mean changes in urine flow, Na and K excretion for each period during and after glucose infusion are shown for early experiments in Fig. 2 and for late (unilateral denervation) experiments in Fig. 3. The effects of bilateral cervical vagotomy on the Na response to glucose infusion are given in Table 1 with the results from early experiments combined with those from the late experiments, innervated kidney only. The difference in response between innervated and denervated kidneys is shown in Fig. 4. Occasional changes which differed from zero could occur by chance despite statistical significance. Hence, only those findings which were reasonably consistent and usually found by both methods of calculation will be considered to be demonstrated. These include pronounced increased excretion of Na seen during second infusion of glucose into HPV innervated kidney by vagotomy and attenuation by renal denervation.

The pattern of response to HPV infusion of glucose was consistent for early as well as late experiments (Fig. 2 and 3). First infusion (early experiments) had no clear effect on



HPV and FEM infusion of glucose test change in urine flow, Na and K excretion during and after the infusion as for HPV infusion same as Fig. 1. All infusions were respectively 5, 4, for 1st, 2nd, and after vagotomy etc.

flow Na or K excretion. Second infusion of glucose increased Na excretion by the R method of calculation (Fig. 1) and during G by the Co to H approach (Fig. 2). In the point when all rats with innervated kidneys were considered, second infusion of glucose significantly increased Na excretion during and for 10 min after infusion (Table I). Vagotomy eliminated the increase in Na excretion during glucose infusion and caused a significant decrease following infusion (Table I). The differences in responses before and after vagotomy were significant for all four periods. G, G₂, R, and R₂. The response of the denervated kidney is shown in Fig. 4 and applied to both Na and K excretion. The N value in the figure is the number of observations before and after vagotomy. The results were equally significant if all comparisons on a given rat were averaged and N was reduced to the number of rats.

The pattern of response to FEM infusion of glucose was not the same for early and late experiments. In the early experiments, first infusion of glucose decreased urine flow and excretion during the infusion and Na and K excretion afterwards (Fig. 2). In the late experiments, there was a trend for first infusion to increase water and electrolyte excretion during the infusion which became significant in innervated kidneys after vagotomy (Fig. 3 and Table I). Note that vagotomy had the opposite effect to that seen with HPV infusions. Renal denervation may have depressed the increase in K excretion but had no effect on the Na excretion response (Fig. 3 and 4).

In the majority of experiments arterial blood pressure was unchanged by HPV or FEM infusion of glucose. In two experiments, HPV glucose did increase blood pressure by 10 to

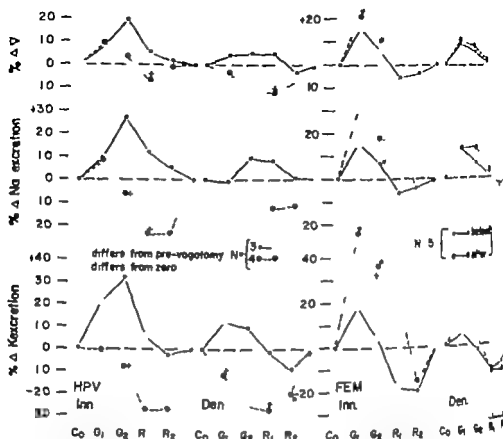


Fig. 3 HPV and FEM infusions of glucose on percent changes in urine flow, Na and K excretion and left kidneys following denervation of the left kidney. The 3 pre-vagotomy HPV infusions are second infusions and the 3 pre-vagotomy FEM infusions were all first infusions.

15 mmHg and slug injections of glucose into HPV (after urine collections had been completed) almost invariably increased blood pressure as noted by Schmidt (1973). Vagotomy increased blood pressure by 10 to 25 mmHg during the procedure and for 15 min thereafter. Later pressure gradually fell and experiments were terminated if pressure fell below 95 mmHg.

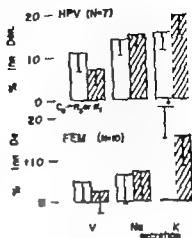
Frequent testing of urine for glucose during infusion invariably gave negative results.

TABLE I Percent change in Na excretion during and after infusion of glucose into HPV and FEM and after vagotomy

		(N)	G	G change mean \pm S.E.	R	R ₂
HPV	before	(10)	12.5 \pm 4.7 ^a	20.1 \pm 7.1	9.7 \pm 4.2 ^a	4.1 \pm 4.1
	after	(9)	-3.3 \pm 4.2 ^b	-3.1 \pm 2.3 ^b	-16.8 \pm 5.1 ^{a, b}	-17.3 \pm 4.1
FEM	before	(9)	8.6 \pm 5.9	4.3 \pm 2.2	-4.3 \pm 3.6	-17.3 \pm 4.1
	after	(10)	22.7 \pm 9.4 ^a	17.4 \pm 6.3 ^a	9.1 \pm 7.9	-7.5 \pm 4.6

^a Differs from zero.

^b Differs from pre-vagotomy.



Percent change in functions of the innervated kidney (of the denervated kidney (paired kidneys) calculated the mean change during glucose infusion by both C_0 to R_1 and C_0 to R_2 interpolations. Values in flow, Na and K excretion are given for HPV and sham. A double asterisk indicates significance at probability level.

Discussion

Our studies in unanesthetized rats (Blake and Lin 1978) had shown that HPV infusion of glucose at 1 ml/h for 30 min increased saline and decreased H_2O intake. The purpose of the present studies was to determine if HPV infusion of glucose had an effect on Na excretion by the kidney that could be attributed to receptors located in the hepatic portal vein. The rate of glucose infusion was the same as in the previous studies (1 M glucose at 1 ml/h) would not have increased plasma glucose concentration to more than 160 to 180 mg/dl (Booth and Jarman 1976), well below the threshold for glycosuria. Consequently, diuresis could not be attributed to an osmotic diuresis from glucose excretion. The results were clear. Second infusion of glucose into HPV increased Na excretion prior to surgery but not afterwards. An additional observation was that renal denervation attenuated the response to HPV glucose during the time of infusion but not afterward. After HPV infusion decreased Na and K excretion following the period of infusion. Infusions of glucose tended to evoke a delayed decrease in Na and K excretion before surgery but after surgery Na and K excretion were increased during glucose infusion but significantly decreased afterwards.

A possible explanation of the results is not obvious. The following speculation is offered as the most parsimonious postulate but must certainly be considered to be overhyped and incomplete. There might be three receptors sensitive to glucose: central, splanchnic sympathetic afferents, and hepatic vagal afferents. There is good evidence for such receptors in the literature (Oomura *et al.* 1969; Schmitt 1973). FEM infusion would be expected to activate the central receptors more than the peripheral and HPV infusion would activate the peripheral more than the central, especially first infusion of glucose. Second or more HPV infusion of glucose (the liver now saturated) would be expected also to reach the central receptors. Both central and vagal receptors would tend to increase Na excretion, the latter by blocking neurally mediated decreases considered by some and operative procedures, see for example, Blake (1953). The splanchnic

glucose receptors would also tend to decrease Na excretion. Thus, FEM infusion second would have little consistent effect, sometimes increasing sometimes depending on balance of central stimulatory and splanchnic inhibitory effects. HPV infusion would be similarly uncertain because of opposing vagal and splanchnic effects. HPV second infusion by adding stimulation of central receptors to that of vagal would lead to increased Na excretion. Vagotomy would eliminate the increase but have little effect on the FEM infusion response. The tendency for Na excretion to increase more after vagotomy with FEM infusion might be the result of inhibition from other vagal afferents, e.g. from cardiopulmonary receptors.

One difficulty with accepting central receptors as playing a role are the studies of and Kaley (1976). They found that infusing 2.5 M glucose into the carotid artery had no significant effect on Na excretion. However the dogs received sufficient doses of vasopressin and 9 α fluorohydrocortisone to eliminate effects mediated via these hormones. In conscious goats, infusion of hypertonic glucose into the carotid artery (arterial) has a weak antidiuretic effect presumably due to release of some antidiuretic hormone (Eriksson, Fernandez and Olson 1971) and Buckalew and Dimond (1976) have postulated that vasopressin is natriuretic by virtue of releasing a natriuretic hormone. This effect might account for some of the increased Na excretion observed in the present studies not for that fraction of the increase resulting from inhibition of neurally-mediated decrease in Na excretion. Inhibition of the neurally-mediated decrease was seen only during HPV glucose infusion and not during FEM infusion. These observations, therefore, do not confirm a dual effect: a central natriuretic effect and a vagally-mediated inhibition sympathetically mediated decrease in Na excretion.

The delayed decreases in Na and K excretion, pre-vagotomy with early FEM infusion and post vagotomy with HPV infusion, could not be attributed to enhanced renal activity. It is possible that these late decreases were the result of insulin secretion (Lefebvre and Crabbé 1971) but we have no evidence to support such a conclusion.

In brief, glucose infusion appeared to have conflicting effects on Na excretion with end result being dependent on variable activation of several different receptors. Central vagotomy converted an increase in Na excretion during HPV glucose to a decrease following the infusion but had no effect on the variable response to FEM infusion.

References

- BLAKE, W. D. Some effects of dihydrogenated ergot alkaloids on renal hemodynamics, water and electrolyte excretion in the dog. *Amer. J. Physiol.* 1953, 173, 337-344.
- BLAKE, W. D. and K. K. LIN, Hepatic portal vein infusion of glucose and sodium solutions and effect of saline drinking in the rat. *J. Physiol. (Lond.)* 1978, 274, 129-139.
- BOOTH, D. A. and S. P. JARMAN, Inhibition of food intake in the rat following complete absorption of glucose delivered into the stomach, intestine, liver. *J. Physiol. (Lond.)* 1976, 259, 501-512.
- BUCKALEW, V. M. JR. and K. A. DIMOND, Effect of vasopressin on sodium excretion and plasma natriuretic activity in the dog. *Amer. J. Physiol.* 1976, 231, 28-33.
- DALY, J. J., J. W. ROW and P. HERRICKS, A comparison of sodium excretion following the administration of saline into systemic and portal veins in the dog: evidence for a hepatic role in the control of Na excretion. *Clin. Sci.* 1967, 33, 481-487.

- SON, L. O. FERNANDEZ and K. OLSSON, Differences in the antidiuretic response to intracarotid infusion of various hypertonic solutions in the conscious goat. *Acta physiol. scand.* 1971. **83** 554-562.
- WALSH, A. Afferent impulse discharges from glucosensors in the liver of the guinea pig. *Ann. N.Y. Acad. Sci.* 1969. **157** (Art. 2) 690-700.
- WATSON, A. P. and J. CRANDALL, Control by angiotensin of sodium, potassium, and water excretion by isolated dog kidney. *Pflug. Arch. ges. Physiol.* 1971. **323** 11-20.
- WILSON, D. J. D., E. ANDERSON and D. A. VANDERWIELE, The effect of isotonic glucose on eating as a function of feeding restriction and infusion rate. *Physiol. Behav.* 1974. **13**, 3-7.
- YAMAGUCHI, Y., T. OHNO, H. OOTAKA and M. J. W. YIP, Glucose and osmosensitive neurons of the rat posterior hypothalamus. *Neurosci. Lett.* 1969. **222**, 282-284.
- ZHANG, S. S., J. R. THOMAS and A. B. ROTHGALLER, Hepatic receptors in control of sodium excretion in anesthetized rats. *Amer. J. Physiol.* 1973. **224**, 373-375.
- ZHANG, M. Influence of hepatic portal receptors on hypothalamic feeding and satiety centers. *Amer. J. Physiol.* 1973. **225**, 1089-1095.
- ZHANG, F. H. and G. KALEY, Natriuresis induced by intracarotid infusion of hypertonic NaCl. *Amer. J. Physiol.* 1976. **232**, 627-633.

Lipoprotein-lipase activity of human skeletal-muscle and adipose tissue after intensive physical exercise

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Abstract

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Seven young, healthy subjects performed bicycle exercise with a workload leading to exhaustion after one hour of work. The tests were done in the afternoon in the fed state. The serum insulin concentration decreased from 22 to 4 mU/l and plasma glucagon increased from 41 to 340 pg/l already after 15 min of work. The level of adipose tissue lipoprotein lipase activity (LPLA) did not fall as had been expected to increase. The skeletal muscle LPLA was unchanged. The results indicate that during the first hour of heavy exercise the heparin-releasable LPLA in tissues is not influenced by the work induced changes in serum hormone levels.

Lipoprotein-lipase activity (LPLA) is regarded to be the rate limiting factor in the transport of the fatty acid component of the serum triglycerides into tissues (Robinson 1970). In man the effect of feeding has been thoroughly studied with regard to adipose tissue LPLA (Nilsson-Ehle 1974, Pykälä et al 1975, Smith and Brunzell 1975, Nilsson-Ehle, Carlström and Belfrage 1975, Lithell *et al* 1978 a) but less so with regard to skeletal-muscle LPLA. However, recently we reported that the LPLA of skeletal muscle tissue was lower in the fed state than in the fasting state during an ordinary day of work (Lithell *et al* 1978 a).

It has been shown in man that the increase of LPLA of adipose tissue in the fed state is dependent on the increment of serum insulin above basal level (Pykälä *et al* 1975, Nilsson-Ehle *et al* 1975). In contrast to this, the skeletal-muscle LPLA has been reported to be inversely related to the fasting serum insulin concentrations (Lithell *et al* 1978 b). Skeletal muscle LPLA in the rat increased after glucagon perfusion *in vitro* (Borenstajn, Kogut and Rubenstein 1973). Studies in man have shown that when the ratio between insulin and glucagon is relatively low as in the fasting state (Lithell *et al* 1978 a) or during diazepam therapy (Lithell *et al* 1978 c) skeletal muscle LPLA is increased.

In fed rats exercise leading to exhaustion after 1 h decreased the LPLA of adipose tissue

Increased LPLA of heart-muscle tissue (Nikkilä, Torsti and Penttilä 1963). As serum lipase decreases and glucose increases during exercise (Böttiger, Faloona and Unger 1971), changes in tissue LPLA may have occurred due to the changed levels of circulating lipase.

In the present study was undertaken to find out whether tissue LPLA in man changed in a certain manner after heavy physical work for 1 h.

Materials and Methods

Subjects (1 female, 6 males) with mean age of 24 years (range 22–28 years) declared themselves as volunteers and consented to take part in the study.

Experimental schedule

The study started at 2 p.m. and the subjects had been doing their ordinary physically light work during the day and had eaten breakfast, lunch and two small meals. Specimens of subcutaneous adipose tissue of the abdomen were taken by needle-biopsy technique (Hirsch and Goldrick 1964) and of skeletal muscle tissue of the m. vastus lateralis as earlier described (Bergström 1962). Blood samples were taken for determination of insulin and glucose.

After the first biopsy the subjects rested for 1 h and then performed ergometer bicycle exercise for 1 h. The work load was chosen so that the subjects were totally exhausted after 1 h of exercise (see Methods section).

At 30 and 60 min of work blood samples were taken for glucose and insulin determination. Three more samples were taken immediately after the exercise.

Analyses

Adipose specimens were stored frozen in liquid nitrogen and the LPLA of the tissue specimens from one subject were later analysed on the same experimental day. The tissue specimens were solubilized at 37°C in 1 ml sodium cholate containing heparin (1 µg) and the LPLA was determined as the release of fatty acids.

Human-activated Lactated caseinogen prepared by Vacker AB, Sweden, using trypsin-trypsin as enzyme (Luthi and Boberg 1977, Luthi and Boberg 1978). The enzyme activity was expressed as the amount of fatty acids (FA) per gram tissue and minute ($\mu\text{mol FA g}^{-1} \text{ min}^{-1}$).

Plasma insulin concentration was analysed in quadruplicate using the Phadebas method (Pharmacia AB, Uppsala, Sweden) based upon the radioimmunoassay technique described by A. A. and Porada (1967). The insulin concentration is expressed as mU-insulin/litre (mU/l). Plasma glucose was analysed according to Unger *et al.* (1970), using glucose oxidase (30 K), specific for pancreatic glucose (Aglar, Parada, Essentios and Unger 1969). The blood samples for glucose analysis were collected in chilled tubes containing heparin and proteinase inhibitor Trasylol (Boehringer, Germany 400 KIU/ml of blood). The specimens were immediately centrifuged and plasma separated and stored at -70°C until assayed. For glucose (Novo, Copenhagen, Denmark) used as standard. Duplicate analyses were made. The maximum work load that could be sustained for 1 h was estimated in each subject on electrically braked ergometer bicycles, beginning at a work load of 100 W and increasing by 100 W every 6 min until exhaustion. W max values ranged between 165 and 200 W in the subjects. The W max value was used to predict the work load that could barely be tolerated for 60 min (W 60) using the formula: $K (\log 6 - \log 60) / (\log W_{\text{max}} - \log W_{60})$, here K is chosen between 5 and 6 according to the degree of physical fitness of the subject (Torstén 1963). The W 60 (less than 60 W) was used between 60 and 65% of W max. The one-hour exercise was performed on mechanically braked ergometer bicycles. The work load was adjusted guided by the heart rate at 6 min and slight further increases were performed during the run in some of the subjects as guided by heart rate and appearance of the subject. At 60 min all subjects were completely exhausted with heart rates above 190.

Statistics Differences between results before, during and after work were tested for statistical significance using the two-tailed t-test (Snedecor and Cochran 1971).

TABLE 1 The lipoprotein lipase activity (LPLA) of adipose and skeletal-muscle tissue before and after exercise for 1 h, the serum insulin and plasma glucagon concentrations before and after 30 and 60 min of exercise (mean \pm S.D.) in 7 healthy subjects are given. Paired *t*-test was used for evaluation of the mean change between zero time and 30 and 60 min resp. and the degree of significance is indicated ($\sim 2p < 0.05$ $\sim 2p < 0.01$).

	Adipose-tissue LPLA nmol FA g ⁻¹ min ⁻¹	Skeletal-muscle LPLA nmol FA g ⁻¹ min ⁻¹	Serum insulin mU/l	Plasma glucagon pg/l
Before work (mean \pm S.D.)	97.4 \pm 25.7	77.2 \pm 8.2	21.7 \pm 13.3	41 \pm 16
After 30 min work (mean \pm S.D.)	—	—	4.2 \pm 2.4 ^a	340 \pm 20 ^a
After 60 min work (mean \pm S.D.)	140.3 \pm 45.0	23.0 \pm 8.5	8 \pm 1.5 ^{ab}	377 \pm 35

Results

The LPLA of adipose tissue increased significantly from 97 to 140 nmol FA g⁻¹ min⁻¹ (Table 1). The mean LPLA of skeletal-muscle tissue was 27 (nmol FA g⁻¹ min⁻¹) and 23 after the physical work ($0.05 < p < 0.10$) (Table 1).

The mean serum insulin concentration being 21.7 mU/l before the physical work increased to 4.2 mU/l after 30 min of work and was 2.8 mU/l at the end of the work (Table 1).

The glucagon concentrations were on the average 241 pg/l before the work started, increased to 340 after 30 and were 377 pg/l at the end of the work (Table 1).

Discussion

The present study showed that in non-fasting subjects performing heavy physical exercise for 1 h the LPLA of adipose tissue increased but not that of muscle tissue. The insulin concentration dropped dramatically during the exercise test but in spite of this the adipose-tissue LPLA increased. In the rat, adipose-tissue LPLA decreased during an equally long test (Nikkilä, Torst and Penttilä 1963). This increase in man may be a delayed effect due to the high insulin concentrations during the 6 h of fed condition preceding the experiment. In fact Pykalisto *et al.* (1975) did not find a significant increase of adipose-tissue LPLA in nutritional experiments until 6 h had passed after breaking the fast. The fall of insulin in the present study to low levels may have been too short to affect the time-dependent system of heparin-releasable LPLA in adipose tissue.

In the skeletal muscle the LPLA did not change. In the rat, only the LPLA of heart muscle increased after exercise, but not that of skeletal muscle (Nikkilä *et al.* 1963, Askesen *et al.* 1972). The ratio between insulin and glucagon concentrations after heavy work should favour an increase of LPLA in skeletal muscle according to the results of earlier studies (Lithell *et al.* 1978 a, Lithell *et al.* 1978 c). It is possible that the LPLA in muscle responds during exercise to other factors than insulin and glucagon. E.g. the muscle contractions may lead to local changes within the fibres of the working muscle causing an increase of muscle LPLA. The present study shows that bicycle exercise leading to exhaustion after 1 h of work does not result in an increase of LPLA of the lateral vastus muscle. How-

preliminary data obtained after several hours of exercise have demonstrated a marked increase in skeletal-muscle LPLA (to be published). It thus seems as if the synthesis of the non-releasable muscle LPLA is time-dependent as is that of adipose-tissue LPLA, or caused by hormonal changes or local environmental factors.

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References

1. AL-PADEA, E., A. M. EISENTRAU and R. H. UNGER. Effects of starvation on plasma pancreatic lipase in normal man. *Diabetes* 1969 18 717-723.
2. E. W. G. L. DOREM, R. L. HUNTON, T. W. SNEYD and R. P. DOWDY. Response of rat tissue lipases to physical training and exercise. *FEBS J* 1972 141 123-129.
3. KROGH, J. Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* 1962 Suppl. 68.
4. SÖDERLUND, J. P. KERO and A. H. ROSENBERG. The role of glucagon in the regulation of myocardial lipoprotein lipase activity. *Scandinavian J. Clin. Lab. Invest.* 1973 53 (3) 603-608.
5. JENSSON, I. O. R. FALDOVA and R. H. UNGER. The effect of intensive physical exercise on pancreatic lipase secretion. *Diabetes* 1971 20 Suppl. 1 339.
6. TILLY, I. and R. B. GOLDBECK. Serial studies on the metabolism of human adipose tissue. I. Lipoproteins of the fatty acid uptake and release in small adipose samples of subcutaneous fat. *J. clin. Invest.* 1974 54 1776-1782.
7. TILLY, I. and J. BOSTO, A method of determining lipoprotein-lipase activity in human adipose tissue. *Am. J. clin. Lab. Invest.* 1977 37 351-361.
8. TILLY, I. and J. BOSTO, Determination of lipoprotein-lipase activity in human skeletal muscle tissue. *Acta biophys.* Acta (Aust.) 1978 53B 58-66.
9. TILLY, I., J. BOSTO, K. HELLERÖD, O. LUNQVIST and B. VIMBY. Lipoprotein-lipase activity in human skeletal muscle and adipose tissue in the fasting and the fed states. *Advances in Lipid Research* 1978 (a) 30 89-94.
10. TILLY, I., R. I. BOSTO, K. HELLERÖD and B. VIMBY. Relationships between the lipoprotein lipase activity of human adipose and skeletal-muscle tissue and the distribution rate of injected intralipid. *Advances in the Biological Sciences*. Ed. H. Porter. Pergamon Press, Oxford, New York, 1978 (b) 389-392.
11. TILLY, I., R. I. BOSTO, K. HELLERÖD, O. LUNQVIST and B. VIMBY. Increase of the lipoprotein-lipase activity in human skeletal muscle during clofibrate administration. *Europ. J. clin. Invest.* 1978 (c) 8 34.
12. TILLY, I., P. TOSTI and O. PENNYL. The effect of exercise on lipoprotein lipase activity of rat heart, adipose tissue and skeletal muscle. *Metabolism* 1963 12 (9) 863-865.
13. SÖDERLUND, J. P. Human lipoprotein lipase activity—comparison of assay methods. *Chin. chim. Acta* 1974 51 283-289.
14. SÖDERLUND, J. P., S. CARLSTRÖM and P. BELFRAGE. Rapid effects of lipoprotein lipase activity in adipose tissue of humans after carbohydrate and lipid intake. Time-course and relation to plasma glycerol, triglyceride and insulin levels. *Scand. J. clin. Lab. Invest.* 1975 35 373-378.
15. GILBERT, O. J., P. H. SMITH and J. D. BRUNZELL. Determinants of human adipose tissue lipoprotein lipase. Effect of diabetes and obesity on basal- and diet-induced activity. *J. clin. Invest.* 1975 56 1108-1117.
16. BRADSHAW, D. S. The function of the plasma triglycerides as fatty acid transport. *Cospectives Biochemistry and Metabolism*. Eds M. Florkin and E. M. Stoll. Elsevier Amsterdam, 1970. 18 51-116.
17. FURBER, G. S. and W. G. COCHRAN. *Statistical methods*. Sixth edition. Iowa State Univ. Press, Amer. 1971.
18. SÖDERLUND, J. P. Assessment of physical capabilities. *Acta physiol. scand.* 1963 58 Suppl. 201.
19. UNGER, R. H., E. AL-PADEA, W. A. MILLER and A. M. EISENTRAU. Studies of pancreatic alpha amylase in normal and diabetic subjects. *J. clin. Invest.* 1970 49 837-848.
20. JENSSON, I., R. ARIN and J. FORAT. Radioimmunosorbent assay of proteins. Chemical couplings of antibodies to insoluble dextran. *Immunochemistry* 1967 4 381-386.

Axonal transport of adrenaline, noradrenaline and phenylethanolamine-N methyl transferase (PNMT) in sympathetic neurons of the cod, *Gadus morhua*

By

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Abstract

ABRAHAMSSON T. Axonal transport of adrenaline, noradrenaline and phenylethanolamine-N methyl transferase (PNMT) in sympathetic neurons of the cod, *Gadus morhua*. *Acta physiol. scand.* 1979 105: 316-325.

The axonal transport of adrenaline, noradrenaline and phenylethanolamine-N-methyl transferase (PNMT) has been studied in holoparasitic neurons of the splanchnic nerve in the cod, *Gadus morhua*. Adrenaline and noradrenaline are transported at a mean axonal transport rate of 16 mm/day. After correction for non-mobile fraction of adrenaline and noradrenaline, which does not contribute to the axonal transport proximal to a ligature, a maximal rate of transport was calculated to about 45 mm/day for catecholamines. The increased level of catecholamines in front of a ligature could be depleted by receptor stimulation, which strongly suggests that both amines are stored in granules. PNMT is transported at a rate of 2 mm/day. The subcellular distribution of the cod PNMT was exclusively non-particulate. The PNMT was further characterized by studying substrate specificity, temperature and pH optima. It is concluded that adrenaline and noradrenaline, stored in granules, are transported in a proximo-distal direction at a high rate compared to PNMT.

Key words: Axonal transport, catecholamines, PNMT, *Gadus morhua*, teleost fish, sympathetic nervous system.

In mammalian sympathetic neurons, axonal transport of noradrenaline and enzymes involved in the synthesis of catecholamines, has been studied by numerous investigators. It has been shown that granular vesicles containing noradrenaline are transported in a proximo-distal direction at a high rate of 5-10 mm/h (Dahlström and Häggendal 1966, Basile 1969, Häggendal *et al.* 1973). This transport rate is similar to that of other granular substances such as dopamine β -hydroxylase (EC 1.14.17.1) (Wooten and Coyle 1973, Brimijoin 1975) and chromogranin A (Geffen *et al.* 1969). Non-granular enzymes such as DOPA-decarboxylase (EC 4.1.1.26) are transported in a proximo-distal direction at a rate of 0.6-3.0 mm/h (Oesch *et al.* 1973, Wooten and Coyle 1973). It has been suggested (Oesch *et al.* 1973) that the rates of axonal transport for the enzymes involved in the synthesis of noradrenaline are well correlated to their subcellular distribution. How-

alloe by the fluorimetric method of Bertler *et al.* (1958) as modified by Håggendal (1961), and separation of tissue extracts on Dowex 50 W X8 resin. The recovery of noradrenaline (100 ng) and adrenaline (100 ng) from the columns was determined and found to be noradrenaline 83 ± 2.1 (s.e.) str. 8 ± 1.6 (n=11). The results given below are corrected for these recovery values.

Phenylethanolamine-N-methyl transferase (PNMT) activity

PNMT was assayed by the method of Axelrod (1962). Nerve segments were homogenized in 2 ml 0.14 M KCl and 100 μ l of the homogenate was incubated at 30°C for 30 min in a total volume of 0.1 ml containing 0.01 M phosphate buffer pH 7.9, 0.4 mM normetanephrine and 2 nmol of ¹⁴C-3-methionine (specific activity 56 mCi/mmole, Amersham Radiochemical Centre). A control incubation in which the substrate (normetanephrine) was omitted was run together with each sample.

The subcellular distribution of PNMT was studied in the celiac ganglion and the splanchnic nerve. Tissue was homogenized in 2 ml ice-cold 0.25 M sucrose and the homogenate was centrifuged at 100 000 g for 15 min. The resulting supernatant was centrifuged at 100 000 g for 60 min to get a "microsomal pellet".

To study the substrate specificity for cod PNMT homogenates of the splanchnic nerve were incubated with a variety of amines at a concentration of 0.4 mM. The ¹⁴C-methyl labelled products were removed into organic solvents as described by Axelrod (1962) and Saeedra *et al.* (1973).

The activity of PNMT is expressed as U/g or mU/cm where one unit (U) equals 1 nmol of ¹⁴C-methyl product formed from the substrate per h.

Fluorescent histochemistry

Segments from the splanchnic nerve were dissected out and quick-frozen, freeze-dried and washed in formaldehyde vapour for 3 h at 80°C as described by Falck and Överman (1965). Sections of 10 μ m were mounted in Entellan (Merck) and viewed in a Leitz Ortholux microscope equipped for fluorescence microscopy.

Statistics

The method of least square was used to calculate regression curves and the Student's t-test for determination of the significance of difference between means (Bailey 1958). Values are expressed as mean \pm S.E.

Results

Temperature and pH optima for PNMT are shown in Fig. 1. The substrate specificity for cod PNMT is summarized in Table I and shows that phenylethanolamine and normetanephrine were substrates for cod PNMT while phenylethylamine was not. The distribution of PNMT in various subcellular fractions show that the PNMT activity was relatively

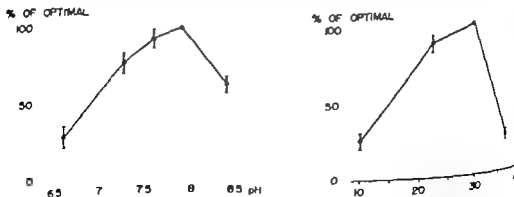


Fig. 1. Effects of pH and temperature on the activity of cod PNMT in the celiac ganglion/splanchnic nerve. The enzyme activity is expressed as per cent of optimal activity in each series of expts. Number of determinations at each point = 6.

1. The substrate specificity for cod N-methyl transferase. The results are mean values expressed as % of activity obtained with phenylethanolamine as substrate. Number of experiments = 4

N-methyl transferase activity	
phenylethanolamine	100
norepinephrine	1
adrenaline	9
catecholamine	79
PNMT	1

in the non-particulate fraction both in ganglion cellaxum and splanchnic nerve (II).

Anatomy of the splanchnic nerve trunk is very different from one animal to another. In some animals the nerve splits up into 2 or 3 parallel trunks which persistently (Stannius 1849 Nilsson 1972). A histochemical study of the cod splanchnic (outside the celiac ganglion) revealed the presence of a very small amount of fluorescent cells which occasionally could be seen in small clusters (Fig. 2 a). The cod splanchnic nerve was ligated for periods of 1-6 days. The fluorescent histochemical study showed large amounts of strongly fluorescent material proximal to the nerve after 3 days (Fig. 2 b) indicating that catecholamines are transported in a proximal direction. The accumulation of adrenaline and noradrenaline proximal to a ligature was rapid and linear for the first 3 days (Fig. 3). Reserpine, given at a time when a certain amount of adrenaline and noradrenaline already have accumulated above the ligature, led to a highly significant reduction of catecholamine content proximal to the ligature, after 3 days after ligation (Fig. 4). PNMT which was also found to accumulate above the ligature, shows a quite different pattern, with a slow and linear accumulation during the 6 day period (Fig. 5). Regression curves were calculated for the linear portion of the accumulation proximal to the ligature and from the slope of each regression line a mean rate of axonal transport was estimated: adrenaline 16.5 mm/day noradrenaline 15.9 mm/day and PNMT 1 mm/day. To estimate the absolute rates of transport for the rapidly accumulating substances, the proportion of adrenaline and noradrenaline free to move distal to the ligature was calculated. The catecholamine content in the 1 cm segment which begins distal to the ligature was measured the 1st and 3rd day after ligation. The results

2. Subcellular distribution of cod PNMT in celiac ganglion and splanchnic nerve. The results are mean values expressed as U/g tissue. Number of experiments = 3.

cellular fraction	PNMT activity	
	Celiac ganglion	Splanchnic nerve
400 g sediment	0.1 ± 0.1	0.1 ± 0.1
600 g sediment	0.2 ± 0.1	0.1 ± 0.1
1000 g supernatant	16.3 ± 0.9	13.7 ± 2.5



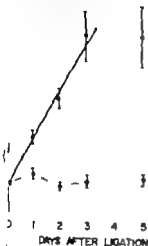
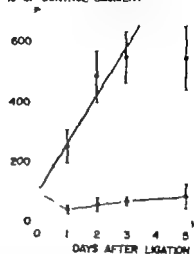
Fig. — Fluorescent histochemical microphotographs showing (A) the presence of fluorescent ganglion cells in the splanchnic nerve (outside the celiac ganglion) and (B) the accumulation of catecholamines proximal to a ligature (arrow) of the splanchnic nerve. Calibration in A = 30 μ m and B = 100 μ m.

(Fig. 6) show that the stationary portion for both adrenaline and noradrenaline is approximately 60–65%. The estimated absolute rates of catecholamine transport would thus be adrenaline 46 mm/day and noradrenaline 42 mm/day at a temperature of 10°C.

Discussion

The disappearance of only a fraction of adrenaline and noradrenaline distal to a ligature shows the presence of a non-mobile fraction which does not contribute to the catecholamine accumulation above a ligature. Thus, in order to calculate the true rapid axonal transport rate, correction for the non mobile fraction must be considered (Häggendal *et al.* 1976). After this correction it was found that both adrenaline and noradrenaline are transported in a proximo-distal direction at a rate in the order of 45 mm/day. The depletion of catecholamines above the ligature after reserpine treatment strongly indicate that both amines are preferentially stored in granules. The observations suggest that the observed axonal transport of catecholamines in cod sympathetic neurons correspond to the fast axonal transport of noradrenaline storage granules in mammalian sympathetic neurons (Dahlström 1966, Banks and Mayor 1972).

Campbell and Gannon (1976) reported the presence of fluorescent ganglion cells regularly clustered along the splanchnic nerve of *Salmo trutta* and *S. gairdneri*. A very

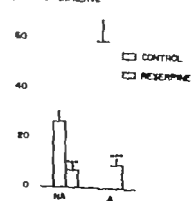
THE CONTENT
CONTROL SEGMENT

 NORADRENALINE CONTENT
% OF CONTROL SEGMENT


the levels in the splanchnic nerve 1 cm proximal to (●—●) and 1 cm distal to (○—○). The adrenaline and noradrenaline content in 1 cm untreated nerve segment at zero time is, adrenaline: 9.6 ± 1.5 μ g ($100 \pm 14.5\%$) ($n = 13$) and noradrenaline: 2.0 ± 0.7 μ g ($100 \pm 14.4\%$) ($n = 13$). All values are expressed as per cent of these control values. Number of experiments at each point = 9–13. Regression lines of accumulation proximal to the ligature were, adrenaline: $Y = 95 \pm 163 X (-0.45)$ noradrenaline: $Y = 110 \pm 159 X (-0.43)$. Correlation coefficients (adrenaline: -0.75 , noradrenaline: -0.70) were significant at $p < 0.001$ for each regression line. Number of exper. = 9.

of fluorescent ganglionic cells were also found in the cod splanchnic nerve, these might store catecholamines and PNMT and it cannot be excluded from this study that these cells in some extent interfere with the nerve segment measurements.

PNMT is transported at a much slower rate than the catecholamines. This rate of flow is similar to that previously reported for PNMT transport in amphibian sciatic nerve (Wooten and Saito 1974). The subcellular distribution of cod PNMT in the splanchnic nerve was

THE AMOUNT ON NERVE



4 Histogram showing the effect of reserpine on the adrenaline and noradrenaline level accumulating in the 1 cm segment proximal to ligature of the splanchnic nerve. The time for ligation was 3 days. Number of experiments = 9. Control and 1 (reserpine) levels of significance: $p < 0.001$.

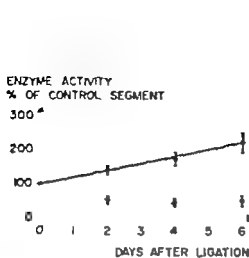


Fig. 5

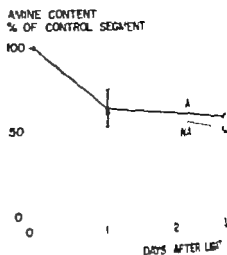


Fig. 6

Fig. 5. PNAIT activity in the splanchnic nerve 1 cm proximal to (●—●) and 1 cm distal to (○—○) ligation. In 1 cm untreated nerve segment at zero time the activity of PNAIT was 5.3 ± 0.6 mU (M.P.) ($n=9$). The values are expressed as per cent of this control value. Number of eyes at each point. Equation for the accumulation period proximal to the ligation was $Y=99+20 X$ (S.E. of Est. Coefficient ($r=0.99$) was significant at $p<0.001$ for the regression line. Number of eyes: 8.

Fig. 6. Adrenaline and noradrenaline content in the 1 cm nerve segment which began 0.5 cm distal to ligation of the splanchnic nerve. All values are expressed as per cent of the content in 1 cm untreated nerve segment at zero time. For absolute values of controls see legend to Fig. 3. Number of eyes at each point. Levels of significance: $p=0.05$, $p=0.01$, $p<0.001$.

exclusively non-particular which is in agreement with the hypothesis that non-particular enzymes are transported at a very slow rate (Oesch *et al.* 1973). However it is not clear from this study that a small fraction of PNMT is transported at a much higher rate. It is not possible to elucidate the problem whether PNMT axonal transport can be separated into different fractions a more accurate method ought to be used (Edström and Hamnerus 1975).

In mammalian peripheral nerve fibers the velocity of rapid axonal transport is 400 mm/day (Ochs 1972, Ochs and Jersild 1974) which is very much faster than the slow rate in this study. McLean and Burnstock (1972) reported that in toad sympathetic neurons the accumulation of adrenaline fluorescence in front of a ligation occurred at a slow rate compared to noradrenaline accumulation in rat sciatic nerve (Dahlgren 1966). In general the fast axonal transport rate in the nervous system of poikilothermic vertebrates are slower compared to mammalian neurons (Grafstein *et al.* 1972, Edström and Hamnerus 1973). Since the fast axonal transport phase is temperature dependent (Edström and Hamnerus 1973) the difference might partly be explained by the circumstances that the experiments on poikilothermic animals are carried out at a temperature below 37°C compared to the optimal temperature for the species examined. In goldfish optic nerve the slow transport rates observed at 20.5°C and 9°C were about 60 mm/day and 70 mm/day respectively (Grafstein *et al.* 1972), the rate at 9°C is similar to the observed fast axonal transport rate at 10°C in cod sympathetic neurons.

results of the present study provide further evidence for the occurrence of PNMT in these neurons of the cod and support the previously presented hypothesis that they can be synthesized in sympathetic neurons and act as neurotransmitter (Abrahamson and Nilsson 1975, 1976). In a preliminary study the pH and temperature optimum of PNMT have previously been reported (Abrahamson and Nilsson 1976). In this study, however, it was shown that the temperature optimum is below that previously reported (37°C). The temperature optimum of 30°C for cod PNMT is similar to the observed one for cod dopamine- β -hydroxylase (Jönsson and Nilsson 1978).

Substrate specificity of cod PNMT in sympathetic neurons for β -hydroxylated phenylethanolamines such as phenylethanolamine and normetanephrine closely correspond to substrate specificity of PNMT obtained from mammalian adrenal medulla and sympathetic nerve and adrenal gland (Axelrod 1962, Wurtman *et al.* 1968, Wooten and Sax 1974). Thus the activity of the N-methylating enzyme measured in cod splanchnic nerve is not due to a non-specific N-methyltransferase of the type previously described in teleost brain (Saravendra *et al.* 1973).

The results from this study confirm the earlier observation that both adrenaline and noradrenaline are stored in an intragranular compartment (Abrahamson and Nilsson 1975). Since this study strongly indicates that PNMT is located in an extragranular compartment, noradrenaline must come out of the granules to be N-methylated to adrenaline. It afterwards returns to the granules to be stored. This would be similar to the presented vesicle pathway for adrenaline from noradrenaline in mammalian adrenal medulla (Axelrod 1962). However Laduron (1972) argued against this translocation mechanism for adrenaline synthesis; he proposed an alternative pathway for adrenaline synthesis via norepinephrine and epinephrine which is taking place inside the granules. Results against this hypothesis presented by Pendleton *et al.* (1976) showed that the noradrenaline level in the mammalian adrenal medulla was increased after PNMT inhibition, indicating that noradrenaline is precursor for adrenaline. Whether noradrenaline or any other amine is the precursor for adrenaline in the cod adrenergic neurons is still to be clarified.

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References

- ABRAHAMSON, T. and E. NILSSON, Effects of nerve sectioning and drugs on the catecholamine content in spleen of the cod, *Gadus morhua*. *Comp. Biochem. Physiol.* 1975, 51C, 231-233.
- ABRAHAMSON, T. and E. NILSSON, Phenylethanolamine-N-methyl transferase (PNMT) activity and catecholamine content in chromaffin tissue and sympathetic neurons in the cod, *Gadus morhua*. *Acta Physiol. Scand.* 1976, 96, 94-99.
- AXELROD, J., T. P. M. GLASSMAN, R. W. MILLARD and M. KING, Regional distribution and subcellular localization of catecholamines in the frog heart. *Comp. Biochem. Physiol.* 1965, 15, 313-324.
- CLARK, J., Purification and properties of phenylethanol-amine N-methyl transferase. *J. Biol. Chem.* 1966, 241, 1657-1660.
- LIT, M. T. J., *Statistical methods in biology*. Hodder and Stoughton 1958.

- BANKS, T. and D. MAYOR, Intra-axonal transport in noradrenergic neurons in the sympathetic system. *Biochem Soc Symp* 1972, 36, 133-149.
- BANKS, P., A. KAPILLER and D. MAYOR, The effects of iproniazid and reserpine on the accumulation of granular vesicles and noradrenaline in constricted adrenergic nerves. *Brit. J. Pharmacol* 1967, 30, 1-10.
- BERTLER, A., A. CARLSSON and F. ROSENQREN, A method for the determination of adrenaline and noradrenaline in tissue. *Acta physiol. scand.* 1958, 44, 73-79.
- BRUNDAGE, S., Stop-flow: a new technique for measuring axonal transport and its application to transport of dopamine- β -hydroxylase. *J. Neurobiol.* 1975, 6, 379-394.
- BRUNDAGE, S. and M. J. WIERMA, Rapid axonal transport of tyrosine hydroxylase in rabbit sciatic nerve. *Brain Res.* 1977, 110, 77-96.
- BURSTOCK, G., Evolution of the autonomic innervation of visceral and cardiovascular systems in vertebrates. *Pharmacol. Rev.* 1969, 21, 47-34.
- CAMPBELL, W. and B. J. GANON, The splanchnic nerve supply to the stomach of the trout, *Salmo gairdneri*. *Comp. Biochem. Physiol.* 1976, 53C, 51-53.
- DAHLSTRÖM, A., Observations on the accumulation of noradrenaline in the proximal and distal peripheral adrenergic nerves after compression. *J. Anat. (Lond.)* 1965, 99, 677-689.
- DAHLSTRÖM, A. and J. HÄGGGENDAL, Studies on the transport and life-span of amine storage material in the adrenergic neuron system. *Acta physiol. scand.* 1966, 67, 78-208.
- EDSTRÖM, A. and M. HANSSON, Temperature effects on fast axonal transport of proteins in rabbit sciatic nerves. *Brain Res.* 1973, 58, 345-354.
- EULER, U. S. v., Adrenergic neurohormones. In *Comparative endocrinology* U. S. v. Euler and B. F. (Eds.), Vol. 1, Academic Press, New York and London 1963.
- EULER, U. S. v. and R. FÄLKE, Catecholamines in nerves and organs of *Myxine phyllonotus*, *Spinibarbus* and *Gadus callarias*. *Gen. comp. Endocr.* 1961, 1, 191-194.
- FÄLKE, B. and C. H. OWMAN, A detailed methodological description of the fluorescence method for cell-by-cell demonstration of biogenic monoamines. *Acta Univ. Lundensis* 1965, 7, Sect. II, 1-23.
- FÄLKE, B., J. HÄGGGENDAL and C. H. OWMAN, The localisation of adrenaline and adrenergic nerves in the trout. *Quart. J. exp. Physiol.* 1963, 48, 353-357.
- GANNON, B. and G. BURSTOCK, Excitatory adrenergic innervation of the fish heart. *Comp. Biochem. Physiol.* 1969, 29, 765-773.
- GEFFEN, L. B., B. G. LIVETT and R. A. RUSH, Immunohistochemical localisation of protein content of catecholamine storage vesicles. *J. Physiol. (Lond.)* 1969, 204, 593-605.
- GRAFSTEIN, B., D. S. FORMAN and B. S. McEWEN, Effects of temperature of axonal transport and rate of protein in goldfish optic system. *Exp. Neurol.* 1972, 34, 158-170.
- HÄGGGENDAL, J., An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissue. *Acta physiol. scand.* 1963, 39, 241-254.
- HÄGGGENDAL, J., A. DAHLSTRÖM and P. A. LARSSON, Rapid transport of noradrenaline in adrenergic nerves of rat sciatic nerve distal to crush. *Acta physiol. scand.* 1975, 94, 386-392.
- HÖFELT, T., K. FUXA, M. GOLDSTEIN and O. JOHANSSON, Immunohistochemical evidence for the existence of dendritic neurons in the rat brain. *Brain Res.* 1974, 66, 233-231.
- JÖNSSON, A.-C. and S. NILSSON, Effects of pH, temperature and Cu^{2+} on the activity of dopamine- β -hydroxylase from the chromaffin tissue of the cod *Gadus morhua*. *Comp. Biochem. Physiol.* 1978, in press.
- KIRSHNER, N. and M. C. GOODALL, The formation of adrenaline from noradrenaline. *Biochem. J.* 1957, 24, 658-659.
- LADURON, P., N-methylating of dopamine to epinephrine in adrenal medulla: A new model for the biosynthesis of adrenaline. *Arch. int. Pharmacodyn.* 1972, 195, 197-208.
- MAZZAUDI, M., Recherches sur la biosynthèse, la sécrétion et le catabolisme de l'adrénaline et de la noradrénaline chez quelques espèces de cyclostomes et de poissons. Thesis, Faculty of Sciences, Paris VI.
- MCLEAN, J. R. and G. BURSTOCK, Axoplasmic flow of dendritic and monoamine oxidase in cranial sympathetic nerves. *Z. Zellforsch.* 1972, 124, 44-56.
- NILSSON, S., Autonomic vasomotor innervation in the gas gland of the swimbladder of a teleost fish (*Merluccius*). *Comp. gen. Pharmacol.* 1972, 3, 371-375.
- NORTH, W. G., Estimation of catecholamines in vertebrate tissues. *Fourth Years Honours Thesis, University of Melbourne* 1965.
- OGH, S., Rate of fast axoplasmic transport in mammalian nerve fibers. *J. Physiol. (Lond.)* 1972, 227, 645-666.

- and R. A. JENSEN, Jr, Fast axoplasmic transport in nonmyelinated mammalian nerve fibers shown across microscopic radioautography *J Neurobiol.* 1974. 5. 373-377.
- F. U. OTTEY and H. TROENCK, Relationship between the rate of axoplasmic transport and sub-cellular distribution of enzymes involved in the synthesis of norrephinephrine. *J Neurochem.* 1973. 20. 1706.
- TOY, K. C., C. KASER and G. GUNER, Studies on adrenal phenylethanolamine N-methyl transferase (MT) with KK&F 64139, selective inhibitor *J Pharmacol exp. Ther.* 1976. 197. 623-632.
- MA, J. M. I. T. COYLE and J. AXELROD, The distribution and properties of the nonspecific N-methyltransferase in brain. *J Neurochem.* 1973. 20. 743-752.
- MA, J. M. M. PALKOVITZ, M. J. BROWNSTEIN and J. AXELROD, Localisation of phenylethanolamine-N-methyltransferase in the rat brain model. *Nature (Lond.)* 1974. 248. 695-696.
- KIL, A. *Das periferische Nervengewebe der Fische*. Stiller Verlag Rostock. 1849.
- CH, G. F. and J. T. COYLE, Axonal transport of catecholamine synthesizing and metabolizing enzymes *J Neurochem.* 1973. 20. 1361-1371.
- CH, G. F. and J. M. SALVEDRA, Axonal transport of phenylethanolamine-N-methyl transferase in fishotic nerve. *J Neurochem.* 1974. 22. 1059-1064.

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The effect of water temperature on the hormonal response to prolonged swimming

By

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Abstract

GALBO H, M E. HOUSTON, N J CHRISTENSEN, J J HOLST, B. NIELSEN, E. NYGAARD and J SUZUKI. The effect of water temperature on the hormonal response to prolonged swimming. *Acta physiol. scand.* 1979 105 326-337.

The relationship between thermoreception, hormonal secretion and muscular activity in man was studied in a swimmer 60 min in 21, 27 and 33°C water at a speed requiring 68% of \dot{V}_{O_2} max (determined in 21°C). Rectal temperature increased in 33°C ($1.3 \pm 0.4^\circ\text{C}$, mean and S.E.) and 27°C ($0.7 \pm 0.1^\circ\text{C}$) and decreased in 21°C expts. ($0.8 \pm 0.3^\circ\text{C}$). Changes in esophageal and muscle temperatures paralleled changes in rectal temperature. Plasma noradrenaline was higher in 33°C than in 27°C expts. and growth hormone, cortisol and glucagon concentrations increased in 27°C and 33°C expts. only ischaemic forearm lactate were uniformly depressed during swimming at the different water temperatures. In 21°C expts. cortisol and adrenaline concentrations were higher than in 27°C expts. \dot{V}_{O_2} , carbohydrate concentrations and plasma lactate were slightly lower in 33°C expts. Plasma glucose decreased slightly and FFA and glycerol concentrations increased identically in all expts. Heart rate increased continuously during swimming in 21°C and 33°C expts., but not in 27°C expts. In conclusion the rise in body temperatures normally observed during exercise enhances the exercise induced increases in the plasma concentrations of noradrenaline, cortisol, growth hormone and glucagon. Decreased body temperatures may elicit catecholamine secretion as a direct consequence of thermoreception. Shivering may account for previously observed decreases in hormone secretion during cold stress but not for increases in cortisol and growth hormone.

Key words. Insulin, glucagon, cortisol, growth hormone, epinephrine, norepinephrine, exercise, shivering, heat, cold.

During prolonged exercise in a neutral thermal environment a progressive, time dependent tachypneic hyperventilation and hypocapnia and a continuous rise in heart rate and splanchnic vasoconstriction take place. This ventilatory (Dempsey *et al.* 1975) and cardiovascular (Rowell 1974) "drift" is accompanied by and probably—at least in part—mediated by gradually rising body temperatures. Also the hormonal response to heavy exercise

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an increase in the concentrations in plasma of catecholamines, glucagon, growth hormone and cortisol and a decrease in insulin concentrations—is accentuated with the time of exercise (Galbo *et al.* 1979). Since the described hormonal changes are similar to those reported often to be associated with heat stress (Collins and Werner 1968, Maher *et al.* 1973, Gale 1973, Rocha *et al.* 1973, George *et al.* 1974, Fraccella *et al.* 1974), the possibility exists that the exercise induced increase in body temperature enhances the hormonal response to exercise, too.

The hormonal response to cold is generally considered as a direct consequence of thermoreception. However, also this response bears resemblance to the hormonal response to exercise (Beum and Porte 1971, Chin *et al.* 1973, Gale 1973, Östman and Sjöstrand 1976, Tedesco *et al.* 1976, Forichon *et al.* 1977, Johnson *et al.* 1977, Usategui *et al.* 1977). Cold exposure may elicit shivering the hormonal changes may in part be secondary to a cold induced increase in muscular activity. To further elucidate the relationship between thermoreception, hormonal secretion and muscular activity we have now studied hormonal responses to prolonged swimming at different water temperatures.





Methods

Eleven male (age 31 (23–41) years (mean and range) and body weight 73 (65–83) kg) volunteered to be subjects after being fully informed as to the nature and risk of the experiments. All experiments took place during the summer, so that both water speed and temperature can be accurately regulated (Åstrand and Rodahl 1977). Before commencing the actual experiments the subjects underwent a number of practice swims at various water velocities to accommodate themselves to swimming in the flume. Furthermore, on all the occasions the subjects had their maximal oxygen uptake ($V_{O_{2max}}$) determined during breast swimming in 27°C water the velocity of which was increased until the swimmer was completely exhausted (Holmér and Bergh 1974). The $V_{O_{2max}}$ achieved by the subjects during graded swimming at 27°C was 3.74 (2.65–4.27) l min⁻¹. The simultaneously determined pulmonary ventilation and heart rate were 118.6 (109.5–125.4) l min⁻¹ and 169 (163–176) beats min⁻¹, respectively.

Actual experiments consisted of breast stroke swimming for 1 h in the flume at a speed calculated to be 65 per cent of the previously determined $V_{O_{2max}}$. At intervals of 1–5 min each subject swam in water at 21°C, 27°C and 33°C, respectively. The sequence of water temperatures was varied among the subjects.

During an overnight fast the subjects reported to the laboratory in the morning. A catheter was inserted approximately 30 cm into the superficial, cubital vein, and a 2 m long manometer line was attached to the catheter. The manometer line and catheter were filled with physiological saline containing heparin (20 and 130 U/ml per litre). Following 15 min rest in the supine position, blood samples were taken (1). Three rectal thermistor and an oesophageal thermistor (in 3 subjects only) were positioned, chest electrodes for the recording of heart rate (HR) were fixed. The temperature in the deltoid and a lateral muscle was measured using needle thermocouples. Thereupon the subjects entered the flume and floated for 9 min in calm water supported by a flotation board. A sample of expired air and a sample (rest 2) were taken upon the end of this rest period. During the subsequent 60 min of swimming blood and gas samples were collected intermittently as depicted in Fig. 1. At the completion of swimming the subjects were quickly out of the water and muscle temperatures were measured again. The recta were wiped with towel and then set for 15 min at room temperature (18–23°C). At the end of this period the last blood sample (post exercise) was drawn. Rectal and oesophageal temperatures as well as heart rate were continuously monitored by means of a manometer system (Danco Electronic). Oxygen uptake was measured with the Douglas bag method.

In agreement with previous findings (Holmér 1972) the subjects in the present study had lower values of maximal oxygen uptake (14%) and of maximal heart rate (10%) during swimming at 27°C than during cold exercise.

 Pre-exercise at 15 min
 Rest in water 9 min
 Swimming at 1 stroke/1.60 min
 Post-exercise at 15 min

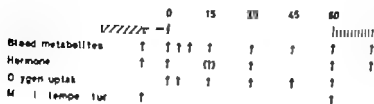


Fig. 1 The sequence of events in a single experiment. The vertical lines indicate the times at which blood and gas were sampled and its temperatures measured.

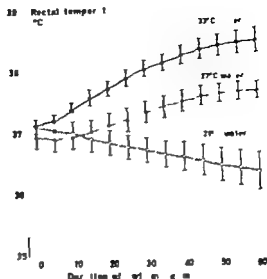
After deproteinization with cold perchloric acid blood was analysed for lactate and glycerol by fluorimetric enzymatic procedures (Lowry and Passonneau 1972). Growth hormone (GH) and cortisol were measured by commercially available solid phase radioimmunoassay kits (Pharmacia CEA-IRE SORIN respectively) 1 U GH equals 0.5 mg of The first international reference preparation of human growth hormone (66/217) from the National Institute for Biological Standards and Control, Huddersfield, London. The intra-assay coefficient of variation of the GH assay was 4% at $24 \mu\text{U}\cdot\text{ml}^{-1}$ and the inter-assay coefficient of variation was 10%. The intra-assay coefficient of variation for the cortisol assay was 13% at $2 \mu\text{g}\cdot 100 \text{ ml}^{-1}$ and the inter-assay coefficient of variation was 15%. Plasma for free fatty acids (FFA), glucose, insulin, glucagon and catecholamines was sampled and analysed as previously described (Galbo *et al.* 1975). Leucine and glucagon were measured by radioimmunoassay. Adrenaline and noradrenaline by a double isotope derivative assay. Every subject had all seven hormones carried out in a single assay run. Statistical evaluation of the data was made by Pearson correlation analysis and by means of the *t* test for paired comparisons. Differences were considered significant if *p* values were less than 0.05.

Results

None of the basal values (rest) differed significantly in the 3 experiments. During rest (rest) oxygen uptake and heart rate were higher in the 21°C expts. ($0.74 (0.52-1.26) \text{ l}\cdot\text{min}^{-1}$ (mean and range) and $73 (54-96) \text{ beats}\cdot\text{min}^{-1}$ respectively) and lower in the 27°C expts. ($0.33 (0.23-0.39)$ and $54 (36-67)$) than in the 33°C expts. ($0.50 (0.32-0.59)$ and $42-78$). At rest, noradrenaline concentrations were always higher than at rest, while the concentrations of adrenaline (59%) (Fig. 3), glucagon (14%) (Fig. 5), and lactate (Fig. 6) only increased significantly upon flotation in the 21°C expts. The remaining values did not change during flotation.

Rectal temperature increased significantly during swimming in the 33°C water ($1.3 \pm 0.4^\circ\text{C}$, mean and S.E.) and to a smaller extent ($p < 0.05$) in the 27°C water ($0.7 \pm 0.1^\circ\text{C}$) but decreased in the 21°C expts. ($0.8 \pm 0.3^\circ\text{C}$) (Fig. 2). In the 3 subjects in whom both rectal and muscle temperatures were measured the changes in rectal and esophageal temperature were identical. Muscle temperatures increased significantly during swimming in the 33°C water (2.6 ± 0.4 and $2.9 \pm 0.3^\circ\text{C}$ in the deltoid and vastus lateralis muscle, respectively) and to a smaller extent ($p < 0.05$) in the 27°C water ($0.5 \pm 0.3^\circ\text{C}$ and $2.1 \pm 0.4^\circ\text{C}$). In the 21°C water the temperature of the deltoid decreased ($1.2 \pm 0.3^\circ\text{C}$) during swimming while that of the vastus lateralis muscle did not change significantly ($+0.6 \pm 0.5^\circ\text{C}$).

The mean oxygen uptake was constant throughout prolonged swimming in the 21°C expts. It averaged $2.22 \pm 0.18 \text{ l}\cdot\text{min}^{-1}$ (33°C-expts.), 2.56 ± 0.24 (27°C-expts.) and $2.51 \text{ l}\cdot\text{min}^{-1}$ (21°C-expts.).



expts.), being significantly lowest in the 33°C-expts., and in the 27°C-water amounting to (59-81)% of the previously determined V_{O_2} max in this environment.

Throughout exercise in all expts. the concentrations of noradrenaline as well as of adrenaline in plasma were significantly higher than at rest (Fig. 3). Compared to the values at 27°C expts. noradrenaline concentrations were significantly higher throughout swimming in the 21°C expts. (on an average 87%) and at the end of exercise in the 33°C expts. (65%). Also the adrenaline concentrations were higher during swimming at 21°C (71%) and at 27°C (71%), the difference being significant between samples obtained at the 30th min of swimming. At that time, although significantly lower than in 21°C expts., adrenaline concentrations in the 33°C expts., too, were significantly higher (25%) than in the 27°C expts. 15 min post exercise the concentrations of catecholamines in the 21°C expts. had not decreased significantly. However in the other expts., they had declined but were still higher than at rest. The mean concentration of noradrenaline was significantly higher in the 33°C expts. than after the 27°C expts.

During and after exercise in the 27°C expts. the concentrations of cortisol in serum increased gradually above values at rest (Fig. 4). In the 33°C expts., compared to the 27°C expts., the concentrations of cortisol increased more rapidly during the first 30 min of swimming. Furthermore, the subjects who had the highest increase in rectal temperature during exercise (from 37.3 to 39.1°C) also had the highest increase in cortisol concentration (from 11 µg/100 ml at rest to 32 and 4 µg/100 ml at the end of and after exercise, respectively). Growth hormone concentrations increased during exercise in 27° and 33°C expts. Above values at rest, the increase being significant after 60 and 30 min of swimming, respectively (Fig. 4). 15 min after exercise in these expts. GH concentrations had returned but were still above values at rest. In the 21°C expts., however neither cortisol nor GH changed significantly during or after exercise.

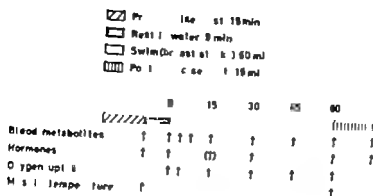


Fig. 1 The sequence of events in a single experiment. The arrows indicate the times at which blood and gas were sampled and rectal temperatures measured.

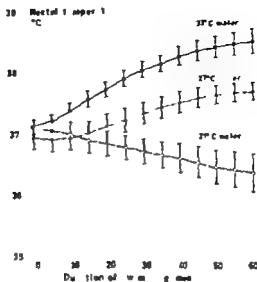
After deproteinization with cold perchloric acid blood was analysed for lactate and phenol red fluorimetric enzymatic procedures (Lowry and Passanelli 1972). Growth hormone (GH) and prolactin (Prl) were measured by commercially available solid phase radioimmunoassay kits (Pharmacia CEA IRE-30RIN respectively). 1 U GH equals 0.5 mg of The first international reference preparation of human growth hormone (66/217) from the National Institute for Biological Standards and Controls, U.K., London. The intra-assay coefficient of variation of the GH assay was 4% at 4 $\mu\text{U ml}^{-1}$ and the inter-assay coefficient of variation was 10%. The intra-assay coefficient of variation for the cortisol assay was 13% at 22 $\mu\text{g 100 ml}^{-1}$ and the inter-assay coefficient of variation was 15%. Plasma for free fatty acids (FFA), glucose, insulin, glucagon and catecholamines was sampled and assayed as previously described (Galbo *et al.* 1975). *Le* Insulin and glucagon were measured by radioimmunoassay and adrenaline and noradrenaline by a double-isotope derivative assay. Every subject had all analyses carried out in a single assay run. Statistical evaluation of the data was made by non-parametric correlation analysis and by means of the *t*-test for paired comparisons. Differences were considered significant if *p* values were less than 0.05.

Results

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The mean oxygen uptake was constant throughout prolonged swimming in the different expts. It averaged 2.22 ± 0.18 l/min (33°C-expts.) 2.56 ± 0.24 (27°C-expts.) and 2.51 l/min (21°C-expts.).



Mean rectal temperatures (\pm S.E., during swimming at 3 water tempera-

tures), being significantly lowest in the 33°C-expts., and in the 27°C-water amounting (59-81)% of the previously determined \dot{V}_{O_2} max in this environment.

During exercise in all expts. the concentrations of *noradrenaline* as well as of *adrenaline* in plasma were significantly higher than at rest, (Fig. 3). Compared to the values in 27°C expts. *noradrenaline* concentrations were significantly higher throughout swimming in the 21°C expts. (on an average 87%) and at the end of exercise in the 33°C (65%). Also the *adrenaline* concentrations were higher during swimming at 21°C and 27°C (71%), the difference being significant between samples obtained at the 30th min of swimming. At that time, although significantly lower than in 21°C expts., *adrenaline* concentrations in the 33°C expts., too, were significantly higher (25%) than in the 27°C

15 min post exercise the concentrations of catecholamines in the 21°C expts. had decreased significantly. However, in the other expts., they had declined but were still higher than at rest. The mean concentration of *noradrenaline* was significantly higher in the 33°C expts. than after the 27°C expts.

During and after exercise in the 27°C expts. the concentrations of *cortisol* in serum increased gradually above values at rest (Fig. 4). In the 33°C expts., compared to the 27°C expts., the concentrations of *cortisol* increased more rapidly during the first 30 min of swimming. Furthermore, the subject who had the highest increase in rectal temperature during exercise (from 37.3 to 39.1°C) also had the highest increase in *cortisol* concentration (from 11 μ g 100 ml⁻¹ at rest to 32 and 42 at the end of and after exercise, respectively).

Plasma *growth hormone* concentrations decreased during exercise in 27° and 33°C expts. below values at rest, the decrease being significant after 60 and 30 min of swimming, respectively (Fig. 4). 15 min after exercise in these expts. GH concentrations had increased but were still above values at rest. In the 21°C expts., however, neither *cortisol* nor GH changed significantly during or after exercise.

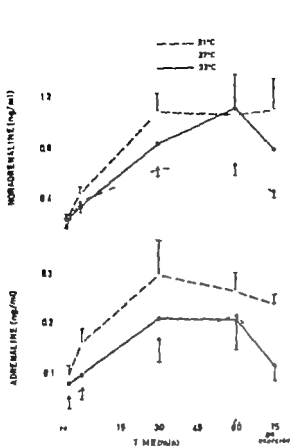


Fig. 3

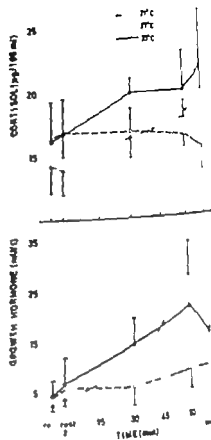


Fig. 4

Fig. 3 Mean concentrations (\pm S.E., $n = 6$) of catecholamines in plasma obtained at rest and during swimming at 3 water temperatures.

Fig. 4 Mean concentrations (\pm S.E., $n = 6$) of control and growth hormone in serum obtained at rest and during swimming at 3 water temperatures.

At the 15'th and 30'th min of swimming in the 27° and 33° C expts. respectively glucose concentrations had significantly decreased below values at rest (Fig. 5). Subsequently glucose concentrations increased and were 15 min post exercise significantly higher than at other time in these expts. In the 21° C expts. glucose concentrations did not decrease significantly during or after exercise. Throughout exercise insulin concentrations in plasma were always significantly below values at rest (Fig. 5). After exercise insulin concentrations increased significantly but remained in the 21° C expts. significantly below rest values. Glucose concentrations in plasma decreased slightly in all expts. during swimming, however being significant only in the 27° C expts. at the end of swimming (Fig. 5). Insulin: glucose molar ratios were uniformly depressed during swimming at the three water temperatures. In the 21° C expts. decreases in glucose concentrations were inversely related to the simultaneous decreases in rectal temperature ($r = -0.90$, $p < 0.01$), and in the 27° and 33° C expts. decreases in glucose concentrations were inversely related to the simultaneous increases in rectal temperature ($r = -0.73$, $p < 0.01$). After exercise in the 33° C expts.



Mean concentrations (\pm S.E. $\times 6$) of glucose, insulin and glycerol in plasma obtained before and during swimming at 3 water temperatures.

³ glucose concentration increased significantly above the mean concentration at the start of swimming but remained in 27°C expts. significantly below preexercise values. ⁴ carbohydrate combustion rate (calculated from O_2 uptake and R value (respiratory quotient ratio) measurements) did not change significantly with the duration of swimming. ⁵ tended to be higher in the 21°C expts. than in the 33°C expts. (representing on an average 0.68 and 0.59 (29.39)%, ($p < 0.1$), respectively of total energy output per min) and was intermediate in the 27°C expts. (53 (34.61)%). The concentrations of FFA in serum decreased at the beginning of swimming but subsequently they increased and were above preexercise concentrations—at the end of and after swimming significantly above preexercise values (Fig. 6). At identical times in the different expts. the concentrations of FFA and of glycerol were identical. In 21°C and 27°C expts. the blood lactate concentrations peaked a maximum early in exercise and were in the 15th min in these expts. significantly

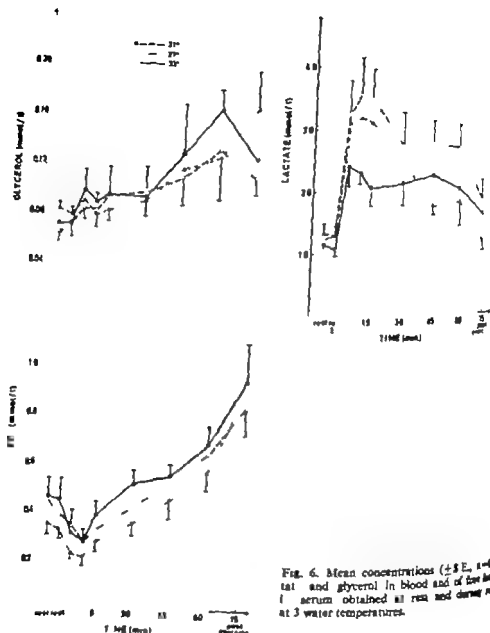


Fig. 6. Mean concentrations (\pm S.E., $n=8$) of lactate and glycerol in blood and of free fatty acids in serum obtained at rest and during swim at 3 water temperatures.

higher than in 33°C expts (Fig. 6). Throughout swimming in 21°C and 33°C expts and in 33°C expts. also after exercise, lactate concentrations were higher than preexercise.

In the 5th min of swimming the mean heart rates were identical in the 3 expts (21°C) 131 ± 5 (27°C) and 127 ± 7 (21°C). Subsequently heart rate increased significantly more at 33°C than at 27°C (to 143 ± 8 and 137 ± 5 respectively in the 60th min). No change occurred at 21°C. Accordingly throughout exercise the oxygen pulse (stroke volume \times heart rate) was significantly lower in the 33°C expts. than in the other expts. and in the final part of exercise also lower in 27°C expts. than in 21°C expts. The subjective experience of swimming at the different water temperatures varied from individual to individual. In general the subjects felt cold in the 21°C water and enclosed in heat in the 33°C water and found it more difficult to complete the swim at these water temperatures than at

Discussion

present study lends support to the concept that the rise in body temperatures normally observed during exercise enhances the exercise-induced increases in the plasma concentrations of noradrenaline, cortisol, growth hormone and glucagon. A wide range of deep as well as superficial body temperatures were attained. Skin temperature has previously been shown to be closely related to water temperature during swimming (Nadel *et al.* 1974) and observed changes in rectal (Fig. 2), esophageal and muscle temperatures, too, varied in parallel with the temperature of the water. The changes in rectal, esophageal and muscle temperatures almost covered the range of changes in these temperatures found during submaximal exercise in a neutral thermal environment (Saltin and Hermansen 1966). When in water the external conditions for heat dissipation were worst the increases in plasma hormone concentrations and heart rate during swimming were larger than those occurring at 27°C. Correspondingly the exercise induced noradrenaline excretion in the urine has been shown to increase in a hot environment (Maher *et al.* 1972), and the concentrations of catecholamines in plasma are higher during running, when secretion of noradrenaline is impeded by atropine (Galbo *et al.* 1977). These findings are in accordance with the opinion (Rowell 1974) that heat stress in addition to exercise elicits an increased sympathetic nervous outflow which in turn increases heart rate and decreases visceral blood flow and thus making possible maintenance of cardiac output and muscular blood flow in the face of increased skin blood volume and flow. It should be emphasized, however, that the plasma noradrenaline level is only an indirect measure of sympathetic activity. Certainly noradrenaline levels in the venous effluent have been shown to be directly related to the sympathetic nervous activity in an organ (Yamaguchi, de Paula and Nadeau 1975). But it is at the present time not possible to exclude the possibility that the relationship between impulse frequency and transmitter overflow to the blood stream is different in different organs and dependent on the physiological milieu (e.g. temperature) at the adrenergic nerve endings (Langer 1974, Starke 1977). Furthermore the elimination of noradrenaline from plasma may vary during different physiological conditions. Thus identical overall activities in the sympathetic nervous system may in theory during different patterns of regional nervous activity and during different physiological conditions lead to different noradrenaline concentrations in arterial plasma. During swimming the concentrations of cortisol and growth hormone in serum only increased in experiments in which body temperature increased (Fig. 4). Furthermore only in such experiments did the post-exercise concentration of glucagon in plasma exceed the preexercise values (Table 1). Available information about the temperature dependence of glucagon secretion is scarce. It solely stems from studies of acute infections in which an elevated body temperature is accompanied by plasma glucagon concentrations which relative to glucose concentrations are increased in the fasting state as well as during intravenous glucose tolerance tests (Rayfield *et al.* 1973, Rocha *et al.* 1973, George *et al.* 1974). In such studies an increased plasma cortisol concentration (George *et al.* 1974), increased urinary free cortisol excretion (Rayfield *et al.* 1973) and an increased serum growth hormone concentration (Rayfield *et al.* 1973) may be found. However the increase in body temperature

may not be essential for the hormonal changes during infection. This is illustrated by the fact that the rise in plasma concentrations of growth hormone (Glick 1963) and glucocorticoids (Collins and Weiner 1968) seen after injection of bacterial pyrogen may occur in the absence of a rise in body temperature. Yet the opinion that increased body temperature enhances cortisol and growth hormone secretion is also based on other kinds of data. Thus cortisol secretion is enhanced when hyperthermia is induced at rest by nalmefene or exposure to a hot environment (Collins and Weiner 1968), and the rise in growth hormone concentration during bicycle exercise has actually been shown to correlate with the rise in rectal temperature (Buckler 1973). It is tempting to propose that increased body temperature also directly stimulates glucagon secretion. Glucose abundance within the pancreatic α cells is possibly the single most potent physiologic glucagon suppressor (Unger and Orci 1976). By increasing the metabolism of glucose an elevated α cell temperature could decrease the suppressive effect on glucagon secretion of prevailing plasma glucose concentrations. In accordance with this hypothesis glucagon concentrations in 33°C expts. in the present study were significantly increased shortly after exercise even though plasma glucose concentrations in these expts. never decreased significantly below preexercise levels. Moreover in two previously studied subjects glucagon concentrations did not increase during prolonged running in a hot environment in spite of an increase in blood glucose concentrations (Rowell *et al.* 1968). Since catecholamines may enhance α cell secretion (Unger and Orci 1976) it could be argued that the temperature dependence of glucagon secretion during exercise is indirect being due to temperature dependent variations in sympathetic activity. However glucagon concentrations did not increase in 21°C expts. (Fig. 5) in which sympathetic activity was at least as intense as in 33°C expts. (Fig. 3). The fact that an increase in the plasma glucagon concentration when at all present was only detectable late in the present expts. is consistent with observations during prolonged running of similar intensity and has tentatively been ascribed to increased hepatic glucose extraction as a consequence of decreased splanchnic blood flow during exercise (Kjeldsen *et al.* 1976).

Immersion of man in 10°C water induces an increase in the concentration of noradrenaline in plasma which is correlated with both the simultaneous increase in metabolic rate caused by shivering and with the degree of central hypothermia (Johnson *et al.* 1977). If the subject is subsequently heated (to approximately 40°C) noradrenaline concentration as well as metabolic rate decreases to basal levels even though core temperature continues to rise (Johnson *et al.* 1977). From these findings it is not possible to decide whether increased release of noradrenaline in response to cold is a direct consequence of thermoreceptor stimulation or secondary to activation of skeletal muscle. However the present study indicates that increased sympathetic activity can be directly elicited from cold receptors. During swimming in 21°C and 27°C water oxygen uptakes were identical but the plasma concentrations of catecholamines in plasma were markedly higher in 21°C expts. in which core temperatures were lower than in 27°C expts. (Fig. 3). A close correlation has previously been found between plasma noradrenaline and heart rate during exercise of more than moderate intensity (Christensen and Brandsborg 1973). This correlation probably reflects the general principle that after vagal withdrawal during exercise increased sympathetic nervous out-

heart to increase its rate is accompanied by a proportional increase in sympathetic motor outflow to visceral organs (Rowell 1974). As expected (Rowell 1974) drift of rate during swimming was eliminated in 21°C expts. in which body temperatures did not increase. Nevertheless the concentrations of catecholamines in these expts. were high during swimming leading to a higher ratio between plasma noradrenaline and heart rate in 27°C expts. This finding questions the predictability of visceral vasomotor tone from heart rate measurements but may rather indicate that during exercise at low body temperatures sympathetic nervous outflow to skin vessels and to the adrenal medulla increases, securing low skin blood flow and volume in co-operation with the direct effects of skin temperature (Irkl *et al.* 1971, Riedel *et al.* 1972, Galo 1973, Rowell 1974). During swimming in 21°C water the concentrations of growth hormone and cortisol in serum did not increase above basal values, neither during mild shivering in the preexercise period nor metabolic rate during swimming was nearly twice as high as previously found during cold shivering (Molnar 1946). These findings indicate that previously observed increases in concentrations of these hormones during cold stress are not exclusively due to shivering. Furthermore, if at all such hormonal responses can be elicited in humans, in order to do so one has to provoke lower body temperatures than those attained in the present study.

Perthermia in the fasting state has been found to be associated with unchanged (George 1974) or decreased (Rayfield *et al.* 1973, Fracchia *et al.* 1977) insulin glucose molar ratios whereas relative hyperinsulinemia has been found after glucose loading (Rayfield 1973, George *et al.* 1974). In markedly shivering adrenalectomized dogs exposed to 21°C ambient temperature rectal temperature decreased 0.8°C (Forichon *et al.* 1977). In these dogs a relative hypoinsulinemia was found during hyperglycemia and tentatively related to an inhibition of insulin secretion by a direct cold-induced stimulation of the autonomic sympathetic nerves (Forichon *et al.* 1977). In the present study plasma insulin concentrations always decreased markedly during swimming (Fig. 5), the insulin glucose ratio being uniformly depressed at the different water temperatures. Thus this study did not reveal any significant influence of body temperatures on insulin secretion during exercise and accordingly does not refute the supposition that the increase in β -adrenergic activity accompanying activation of skeletal muscle is sufficient to explain the depression of insulin secretion found previously during cold stress (Galbo *et al.* 1977).

In agreement with previous findings (Nadel *et al.* 1974) oxygen uptake in the present study was slightly lower during swimming in 33°C water than during swimming at the colder water temperatures. Furthermore, maximal oxygen uptake has previously been shown in man to be directly related to core temperature within the range of core temperatures involved in the present study (Holmér and Bergh 1974). Accordingly the relative work rate ($\dot{V}_{O_2}/\dot{V}_{O_{2\max}}$) possibly varied inversely with water temperature in the latter half of the swimming period. If so differences in relative work load may have influenced the differences in hormonal secretion between the different expts. and may furthermore, explain the tendency to a higher relative carbohydrate combustion rate during swimming in the present study. Whereas the higher blood lactate concentrations in 21°C expts. are compatible with an enhancement of muscular glycogenolysis by the higher plasma adrenaline con-

centration in these expts., identical concentrations of FFA and glycerol and calculated rates of fat combustion in the different expts. do not indicate any inhibition of lipolysis by the higher sympathoadrenomedullary activity in the colder water. Even during swimming in 21°C water lipolysis may have been limited by a low skin blood flow. In conclusion the present study supports the concept that the rise in body temperature normally observed during exercise enhances the exercise-induced increases in the plasma concentrations of noradrenaline, cortisol, growth hormone and glucagon. Furthermore the study indicates that decreased body temperatures may elicit increased concentrations of noradrenaline and adrenaline in plasma as a direct consequence of thermoregulation. Activation of shivering may account for previously observed decreases in insulin secretion during cold stress but do not explain increases in cortisol and growth hormone concentrations.

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References

- ÅSTRAND P-O and S. ENGELHOLM, A swimming fluorine. *J appl Physiol.* 1972. 32: 514
- BAUM, D. and D. PORTZ, JR., Alpha-adrenergic inhibition of immunoreactive insulin release during hypothermia. *Amer J Physiol.* 1973. 225: 303-311
- BUCKLER, J. M. H. The relationship between changes in plasma growth hormone levels and body temperature occurring with exercise in man. *Biomedicine* 1973. 19: 193-197
- CHIM, A. K., R. SEAMAN and M. KAPLESHWARKER. Plasma catecholamine response to exercise at different altitudes. *J appl Physiol.* 1973. 34: 409-412.
- CHRISTENSEN, N. J. and O. BRANDSBO, The relationship between plasma catecholamine concentrations and pulse rate during exercise and standstill. *Europ J clin Invest.* 1973. 3: 299-306
- COLLIER, K. J. and J. S. WEINER, Endocrinological aspects of exposure to high environmental temperatures. *Physiol. Rev.* 1968. 48: 785-839
- DUMPHY, J. A., J. M. THOMPSON, S. C. ALEXANDER, H. V. FORSTER and L. W. CROFT. Respiratory responses on acid base status and their effects on O_2 transport during prolonged muscular work. In: *Adaptation to prolonged physical exercise*. Ed. by H. HOWARD and J. R. POOTMAN. N.S. Brill: Basel. 1973. P. 36-64
- FORNICHON, J. M., J. JOMARY, O. DALLEYET and Y. MENAÏRE, Effect of cold and epinephrine on fat kinetics in dogs. *J appl Physiol.* 1977. 43: 230-237
- FRASCIELLA, M. W., I. S. KAM and B. H. FRANCIS, Metabolic effect of high environmental temperature on non-diabetic and diabetic rats. *Horm Metab Res.* 1977. 9: 374-378.
- GALBO, H., J. J. HOLST and N. J. CHRISTENSEN, Glucagon and plasma catecholamine response to prolonged exercise in man. *J appl Physiol.* 1973. 38: 70-76.
- GALBO, H., N. J. CHRISTENSEN and J. J. HOLST. Catecholamines and pancreatic hormones during β -blockade in exercising man. *Acta physiol scand.* 1977. 101: 428-437
- GALBO, H., J. J. HOLST and N. J. CHRISTENSEN, The effect of depleted carbohydrate stores and of β -blockade on the hormonal response to prolonged exercise. *S. In press.*
- GALBO, H., J. J. HOLST, N. J. CHRISTENSEN and J. HILSTED, Glucagon and plasma catecholamines during β -blockade in exercising man. *J appl Physiol.* 1976. 40: 835-851.
- GALL, C. C., Neuroendocrine aspects of thermoregulation. *Annu. Rev. Physiol.* 1973. 35: 391-408
- GEORGE, D. T., E. J. RAYFIELD and R. W. WANNEMACHER, Altered glucoregulatory hormones in acute pneumococcal sepsis in the rhesus monkey. *Diabetes* 1974. 23: 544-549
- GLICK, S. M. Normal and abnormal secretion of growth hormone. *Ann. N.Y. Acad. Sci.* 1968. 161: 487
- HOLMÉR, I. Oxygen uptake during swimming in man. *J appl Physiol.* 1972. 33: 302-309
- HOLMÉR, I. and U. BRÖNN, Metabolic and thermal response to swimming in water at varying temperature. *J appl Physiol.* 1974. 37: 702-705

- W. REDEL and E. SNOOK, Regional differentiation of sympathetic activity during hypothalamic heating and cooling in anaesthetized rabbits. *Pflügers Arch. ges. Physiol.* 1971. 320 320-331
- L. D. G. J. E. HAYWARD, T. H. JACOB, M. L. COLLIER, J. H. ECKHARTSON and R. H. WILLIAMS, Plasma catecholamine responses of man to cold water. *J. appl. Physiol.* 1977. 43. 216-220
- E. Z. Presynaptic regulation of catecholamine release. *Biochem. Pharmacol.* 1974. 23. 1793-1800.
- H. H. and L. V. PARNON-NEAL, *A flexible system of enzymatic analysis*. New York, Acad. Press.
- J. T. D. E. BARR, D. D. HESTAD, E. T. ANOELLARON and L. H. HARTLEY, Effect of posture on heat production in man. *J. appl. Physiol.* 1972. 33. 8-13.
- G. W. Survival of hypothermia by men immersed in the ocean. *J. Amer. med. Ass.* 1946. 131 1830
- E. R. L. HOUNSLEY, U. BERSON, P.-O. ÅSTRAND and J. A. J. STOLWIJK, Energy exchanges of man. *J. appl. Physiol.* 1974. 36. 465-471
- L. I. and N. O. SÖSTRAND, Reduced urinary noradrenaline excretion during rest, exercise and cold in trained rats - comparison between physically-trained rats, cold-acclimated rats and -non-acclimated rats. *Acta physiol. scand.* 1973. 93. 209-118.
- J. E. J. R. T. CORNWALL, D. T. GROOMER and W. R. BIRNELL, Impaired carbohydrate metabolism in mild and chronic. *New Engl. J. Med.* 1973. 289. 618-621
- W. M. HARRIS and E. SNOOK, Regional differentiation of sympathetic activity during peripheral heating and cooling in anaesthetized rabbits. *Pflügers Arch. ges. Physiol.* 1972. 332. 239-247
- D. M. F. SANTIAPAGO, G. R. FALCONA and R. H. UHLEN, Abnormal pancreatic alpha-cell function in bacterial infections. *New Engl. J. Med.* 1973. 288. 700-703
- L. L. B. HENNING, Cardiovascular adjustments to exercise and thermal stress. *Physiol. Rev.* 1974. 54 59
- L. L. B. H. L. REINIGELMANN, J. R. BLACKMON, R. D. TOWN and F. KOSKOV, Splenic blood flow and metabolism in heat-stressed man. *J. appl. Physiol.* 1968. 26 475-484.
- R. and L. HILLMAN, Esophageal, rectal and muscle temperature during exercise. *J. appl. Physiol.* 1966. 21 1757-1762.
- A. K. Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.* 1977. 77 1-124.
- J. L. K. V. FLATTERY and E. A. SELLERS, Effects of thyroid hormones and cold exposure on release of noradrenaline in cardiac and skeletal muscle. *Can. J. Physiol. Pharmacol.* 1977. 55 515-522.
- A. R. H. and L. ONCI, Physiology and pathophysiology of glucagon. *Physiol. Rev.* 1976. 56 778-826.
- DEY, R., P. GILLER and C. OLIVER, Effect of cold exposure on MSH and ACTH release in the rat. *Neuroendocrinology* 1977. 9 319
- OLSON, N. J. DE CHAMPLAIN and R. NADAR, Correlation between the response of the heart to sympathetic stimulation and the release of endogenous catecholamines into the coronary sinus of the dog. *Am. J. Physiol.* 1973. 226 661-663.

Release of ^3H noradrenaline from the rat vas deferens under various *in vitro* conditions

By

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Abstract

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The release of ^3H -(-)-noradrenaline (NA) from rat vas deferens *in vitro* was examined under experimental conditions. It was found that in normal and reserpinized vas deferens the release of NA by (+)-arbutamine ($5 \cdot 10^{-6}$ M) or low external Na^+ (26 mM) was antagonized by imipramine, desipramine and desipramine inhibitors of the NA uptake, but was not dependent on the presence of Ca^{2+} in the medium and was not antagonized by the potent local anaesthetic agent bethoxycaïne. The release by veratridine in reserpinized tissue was antagonized by the uptake inhibitors but was in general only partially inhibited in presence of Ca^{2+} but almost completely in absence of Ca^{2+} . The release by K⁺ (117 mM) + low Na^+ (26 mM) in normal tissue was dependent on the presence of Ca^{2+} and was antagonized by the muscarinic agonists carbachol and metacholine and by high concentrations of prazosin. In the reserpinized vas the corresponding release was not dependent on Ca^{2+} and was not antagonized by the muscarinic agents but was inhibited by high concentrations of desipramine.

Key words. Noradrenaline release vas deferens, rat, desipramine, imipramine, methiodate.

The impulse-propagated release of noradrenaline (NA) from sympathetic nerve terminals is Ca^{2+} dependent and appears to occur with an exocytotic mechanism (Douglas, Smith and Winkler 1972). Under certain *in vitro* conditions a Ca^{2+} independent NA release mechanism has been demonstrated (Paton 1973). This release is antagonized by cimetidine and desipramine and seems to be a carrier-mediated transport of NA out from the nerve terminals.

In previous studies we found that ^3H -bretylium was released from the rat vas deferens by a Ca^{2+} independent mechanism very similar to the proposed carrier-mediated release of NA (Ross and Kelder 1976a, b, 1977). Since bretylium appears to be accumulated at the NA nerve terminals by the membranal NA carrier (Ross and Gosztonyi 1976, Kelder and Kelder 1976a) it is possible that it is also released by a NA transport mechanism. In order to further establish the similarity between the carrier-mediated release of NA and bretylium in the same organ we have now examined the release of ^3H NA in the rat vas deferens under various *in vitro* conditions.

Materials and methods

of rats deficient weighing about 20 mg from 170-230 g. rats (Sprague Dawley) are used. The incubation medium consisted of 117 mM NaCl, 5.4 mM KCl, 0.6 mM MgSO₄, 2.6 mM CaCl₂, 1.1 mM O₂, 25 mM NaHCO₃, 5.4 mM glucose, 0.25 mM pyridine, 1.1 mM ascorbic acid and 0.13 mM Na₂S₂O₅. In some experiments NaCl was substituted with 250 mM sucrose (low Na⁺) or with 117 mM λ K⁺ (low Na⁺).

Vials were pre-incubated for 15 min in the ordinary incubation medium in order to inhibit the monoamine oxidase (MAO) activity and were loaded with ³H-NA (1 $\times 10^{-6}$ M) by incubation for 1 hour at 37°. The vials were blotted on filter paper and washed three times in fresh incubation medium. The vials were then incubated for 10 min at 37° in order to wash out loosely bound ³H-NA. Each vial of tissue was then transferred to a glass stoppered centrifuge tube containing 0.5 ml of the appropriate incubation medium and the additives to be studied. The tubes were gassed with 93.5% O₂ + 6.5% CO₂. Incubation is performed for 10 min, the vials are blotted on filter paper, weighed and transferred into vials containing 1.0 ml of Soluene-350 (Packard). After one hour at 45° the tissue was dissolved in 1 ml of the scintillation liquid (Perkinblesh III, Packard, in toluene) were added. Six ml of Instagel (Beckman) was added to the incubation medium. The radioactivity was counted in a liquid spectrometer. The radioactivity added to the medium and tissue dissolved in Soluene-350 were determined in each experiment. Percent of radioactivity released into the medium was calculated and expressed as per cent of the activity at the start of the release experiment (i.e. the sum of the radioactivity in tissue and medium). In some experiments the amounts of intact ³H-NA, of ³H-labelled acid and neutral metabolites, and of ³H-metamorphs in the incubation medium were determined by chromatography on weak acid ion exchange resin (Amberlite CG150 Type I (100-200 mesh), Na⁺ 30-5 mm columns), prepared according to Mithrie (1964). Immediately after incubation 2 ml of 0.1N HCl containing 0.05 EDTA Na₂ were added to the media. Vials were stored at -18°C until the assay (this one week). 10 ml of 0.1N Na₂PO₄, 1 ml of 0.1N sodium phosphate buffer pH 6.5 were then added and pH was brought to 6.0 by addition of 0.01N NaOH. 10 ml of this solution were run through the resin column and 1 ml was for determination of total radioactivity. The effluent contained the acid and neutral metabolites. The column was then washed with 2-10 ml of distilled water. ³H-NA was eluted with 10 ml of 4% boric acid. The column was then washed with 10 ml of distilled water and ³H-metamorphs was eluted with 10 ml of 4N NH₄OH. Radioactivity in 5 ml of these fractions was measured after addition of 5 ml of Instagel.

Materials: (*S*)-Noradrenaline-7 H (specific activity 3.3 Ci/mmole) as purchased from NEN GmbH, Boston, Mass. Veratridine was purchased from EGA-Chemie K.G., Steinheim, Albeck, Germany and pyridine from Fisher Laboratories Inc., Morton Grove, Ill., U.S.A. Imipramine was synthesized from norepinephrine, both like desipramine are gifts from Ciba-Geigy A.G. Mefenazine hydrochloride (Mefenazine) is a gift from Les Laboratoires Miliot, Arson, France.

Results

Spontaneous release in reserpinized tissue

Spontaneous release measured as the radioactivity in the medium was markedly temperature dependent (Fig. 1). The release at 37° followed a biphasic course with a rapid initial phase during the first 5-10 min followed by a second slow phase ($t_{1/2} = 2.5 \pm 0.1$ hours). Extrapolation of the second phase to zero time estimates the amount released in the initial phase (10-6 \pm 0.6, of the total radioactivity).

Imipramine (3 $\times 10^{-6}$ M) and imipramine methiodide (2.4 $\times 10^{-6}$ M) accelerated the spontaneous release but antagonized the release evoked by (*S*)-amphetamine (5 $\times 10^{-6}$ M) or veratridine (5 $\times 10^{-6}$ M) (Fig. 1). High concentration of K⁺ (75 mM), low concentration of Na⁺ (26 mM) and high K⁺ (117 mM) + low Na⁺ (26 mM) induced marked release which like that evoked by amphetamine and veratridine was almost completely abolished.

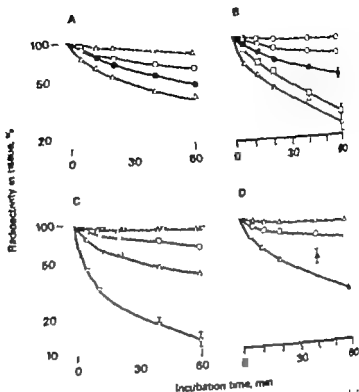


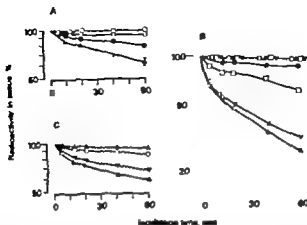
Fig. 1 Time courses of the release of ^3H NA evoked by various means from reserpinized tissue. Each value is the mean \pm S.E. (vertical bars) measured as the radioactivity remaining in the tissue. Each value is the mean \pm S.E. (vertical bars) of 4 determinations. The incubations were performed at 37° (solid lines) or 0° (dotted lines). Control (O). A. Desipramine 3×10^{-6} M (\bullet), ($+$)-Amphetamine, 5×10^{-4} M (Δ), Imipramine methiodide, 2.4×10^{-4} M (\circ), veratridine 5×10^{-5} M (\triangle), Imipramine methiodide, 2.4×10^{-4} M (\square). C. Hypertonic K^+ 75 mM (Δ), high K (117 mM) + low Na (26 mM) (\circ), veratridine 5×10^{-5} M (\square). D. Low Na (26 mM) substituted with 0.25 M sucrose (Δ).

Time course in normal tissue

The spontaneous release of ^3H -noradrenaline from the normal (non-reserpinized) vesicles was much less than that from the reserpinized tissue (Fig. 2). Thus, approximately 10% of the radioactivity accumulated was released during one hour's incubation at 37° . Desipramine (3×10^{-6} M) and Imipramine methiodide (2.4×10^{-4} M) only slightly accelerated this release. ($+$)-Amphetamine (5×10^{-4} M), high external concentration of K (75 mM), low Na (26 mM) and particularly high K (117 mM) + low Na (26 mM) and veratridine (5×10^{-5} M) evoked marked release at 37° but not at 0° .

Dose response at the release evoked by ($+$)-amphetamine

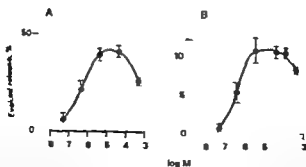
The concentration curve of the release of ^3H NA evoked by ($+$)-amphetamine in the reserpinized tissue showed an optimal effect at the concentration between 1×10^{-4} and 5×10^{-4} M at which about 40 per cent of the accumulated ^3H NA was released (Fig. 3). This curve was almost identical with that observed for the release of ^3H -bretylium (Ross and Kelder, 1976a) and for that of ^3H NA in normal vesicles (Fig. 3). However, the amount released in the normal preparation was only one fourth of that in the reserpinized vesicles. 50 per cent of the optimal release was obtained at about 5×10^{-5} M of ($+$)-amphetamine in three cases.



2. Time courses of the release of ^3H NA evoked by various means from rat vesicles measured as radioactivity remaining in the tissue. Each value is the mean \pm S.E. (vertical bars obviously hidden in the dots) of 4 determinations. Control (O). A. Desipramine, 3×10^{-5} M (●), (+)-amphetamine, 5×10^{-4} M (◐). B. Reserpine methiodide, 2.3×10^{-5} M (●), hypertonic (75 mM) K⁺ (◐), high K⁺ (117 mM) + low Cl⁻ (◑), veratridine, 5×10^{-5} M (◒). C. Low Na⁺ (26 mM, substituted with 0.25 M sucrose) (◐), reserpine methiodide 2.3×10^{-5} M + low Na⁺ (◒).

Separation of H-NA from labelled parabiotics

In order to verify that the radioactivity released into the medium consisted mainly of H-NA, ^3H -labelled neutral and acid metabolites and ^3H -normetanephrine (NM) were separated on ^3H -NA on Amberlite CG50 columns (Table I). No detectable amounts of neutral and acid metabolites were released from the normal tissue and low concentrations were found in the medium of the reserpinized vesicles. The MAO inhibition was accordingly effective. The NM values were low in the medium of the normal tissue but constituted a considerable part of the spontaneous released radioactivity from the reserpinized tissue. However, it was slightly increased by the means which strongly released H-NA into the medium. Hence a evoked release of radioactivity was mainly H-NA.



3. Release of ^3H -NA from reserpinized (A) and normal (B) rat vesicles evoked by various concentrations of (+)-amphetamine and measured as the radioactivity released into the medium in per cent of the total radioactivity in the tissue at the start of the incubation. Each value is the mean \pm S.E. (vertical bars of 4 determinations). The incubation time 10 min.

TABLE I Amounts of ^3H noradrenaline (NA), ^3H -normetanephrine (NM) and ^3H -acid + neutral metabolites (Acid) released from the rat vas deferens under various *in vitro* conditions. The vasa were loaded with ^3H NA and the release of ^3H NA and ^3H -metabolites in the medium during 10 min incubation was determined. The metabolites were separated from NA on Amberlite CGM column. Each value is the mean \pm S.E. of 4 determinations. The neutral and acid metabolites in the rat tissue were below detectable amounts.

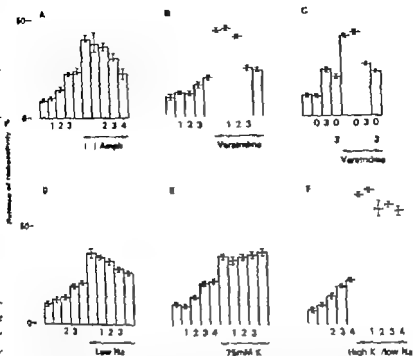
Treatment	Normal tissue		Reserpinized tissue		
	^3H NA	^3H NM	^3H NA	^3H NM	Acid
	pmol/g wet tissue \pm S.E.				
Control	64 \pm 14	9 \pm 2	64 \pm 5	40 \pm 5	5 \pm 4
(+)-Amphetamine 5 \cdot 10 $^{-6}$ M	190 \pm 28	9 \pm 1	430 \pm 6	53 \pm 6	9 \pm 1
Low Na (sucrose)	137 \pm 21	10 \pm 2	273 \pm 10	72 \pm 9	11 \pm 1
High K + low Na	700 \pm 50	6 \pm 4	657 \pm 14	63 \pm 7	11 \pm 1
Veratridine 5 \cdot 10 $^{-6}$ M	629 \pm 31	2 \pm 2	—	—	—

Antagonism of the evoked release by uptake inhibitors

In a series of experiments the antagonism of the evoked release by various compounds was examined. To avoid the initial spontaneous release the vasa were pre-washed for 10 min at 37 $^{\circ}\text{C}$. The release of radioactivity during a further 10 min period was measured. Imipramine methiodide was used besides desipramine as an inhibitor of the membrane NA transport, since it as a quaternary ammonium derivative can be expected to preferably act on the outside of the membranes which minimizes intraneuronal interactions. Betboxycaine was used as a potent membrane stabilizing agent which has weak inhibitory action on the noradrenaline transport (Ross and Kelder 1976 b).

Imipramine methiodide antagonized the release of radioactivity evoked by (+)-amphetamine (5 \cdot 10 $^{-6}$ M) and low external Na $^{+}$ (26 mM) in reserpinized (Fig. 4) and normal vasa (Fig. 5). The release by veratridine (5 \cdot 10 $^{-6}$ M) was only partially (15%) antagonized in the normal tissue but almost completely antagonized by imipramine methiodide in the reserpinized vasa. These findings agree with those previously obtained with desipramine (Ross and Kelder 1976 b). The release evoked by high external K $^{+}$ (75 mM), low external Na $^{+}$ (26 mM) and high K $^{+}$ (117 mM) low Na $^{+}$ (26 mM) was not apparently antagonized by imipramine methiodide (Fig. 4 and 5). Since high concentrations of imipramine methiodide itself caused significant release particularly in the reserpinized tissue, a partial antagonism is possible, if additive effects are assumed.

Desipramine had similar effects but was considerably more potent than imipramine methiodide (Table II), which is in accord with their potencies as uptake inhibitors (K $_{\text{IC}_{50}}$ in the rat vas deferens was 1.1 \cdot 10 $^{-6}$ M for desipramine and 2.3 \cdot 10 $^{-6}$ M for imipramine methiodide). Large concentrations of desipramine antagonized the release of radioactivity evoked by high K $^{+}$ + low Na $^{+}$ in the normal and reserpinized tissues (Fig. 6). These concentrations were 100 times larger than those antagonizing the veratridine evoked release in reserpinized vasa but equal to those effective against veratridine in normal tissue. As shown in Table II the two uptake inhibitors antagonized the release evoked by (+)-amphetamine, low Na $^{+}$ and veratridine in reserpinized vasa at the same concentrations as they inhibited the accumulation of ^3H NA.



4 Antagonism by imipramine methiodide of the release of ^3H -NA from reserpinized rat ves deferens led by various stimuli. The concentrations of imipramine methiodide were: 1- 2.4×10^{-6} M, 2- 2.4×10^{-6} M, 3- 2.4×10^{-6} M, 4- 2.4×10^{-6} M. A. ()-Amphetamines, 5×10^{-6} M. B. Veratridine, 5×10^{-6} M. C. absence of Ca^{2+} and presence of 1 mM EGTA in the incubation medium. Low Na^+ (26 mM) in the medium (substituted with 0.25 M sucrose). E. Hyperosmotic K $^+$ (75 mM) in the medium. F. High K $^+$ (117 mM) low Na^+ (26 mM) in the medium. * denotes significant ($p < 0.05$) difference from the control values (— the releasing agent alone).

Antagonism. The local anaesthetic agent bethoxycaïne did not antagonize the release evoked by ()-amphetamine (5×10^{-6} M), low external Na^+ (26 mM) and high external K^+ (75 mM) in normal or reserpinized tissue (Table III). However the release evoked by veratridine was previously shown to be completely antagonized by bethoxycaïne (Ross & Kelder 1976 b). The release evoked by high K^+ + low Na^+ in normal tissue was slightly (*) but significantly antagonized by bethoxycaïne (Table III).

Ca^{2+} dependence of the release. In order to differentiate between exocytotic and carrier induced release Ca^{2+} was omitted in the incubation medium to which was added 1 mM EDTA to chelate any remaining Ca^{2+} . The release by ()-amphetamine and that by low Na^+ were not influenced by omission of Ca^{2+} (Table IV). In the normal tissue the release by high K^+ and by high K^+ + low Na^+ were almost completely antagonized by omission of Ca^{2+} . The release by veratridine in normal tissue was partially (40%) antagonized by omission of Ca^{2+} (Fig. 5 C). Addition of imipramine methiodide to the Ca^{2+} free medium completely contracted the veratridine evoked release.

In the reserpinized tissue the release by high K^+ + low Na^+ was decreased (1%) by omission of Ca^{2+} (Table IV). No effect was obtained on the ^3H -NA release evoked by the other means studied.

TABLE 1 Amounts of ^3H noradrenaline (NA), ^3H -normetanephrine (NM) and ^3H -acid + neutral metabolites (Acid) released from the rat vas deferens under various *in vitro* conditions. The vas was incubated with ^3H NA and the release of ^3H NA and ^3H -metabolites in the medium during 10 min incubation was determined. The metabolites were separated from NA on Amberlite CG30 silica. Each value is the mean \pm S.E. of 4 determinations. The neutral and acid metabolites in the vessel tissue were below detectable amounts.

Treatment	Normal tissue		Reserpinized tissue		
	^3H NA	^3H NM	^3H NA	^3H -NM	^3H -Acid
	pmol/g wet tissue \pm S.E.				
Control	64 ± 14	9 ± 2	64 ± 5	40 ± 5	5.4
(+)-Amphetamine $5 \cdot 10^{-6}$ M	190 ± 28	9 ± 1	430 ± 6	53 ± 6	9.1
Low Na (sucrose)	137 ± 1	10 ± 2	273 ± 10	7 ± 9	11.1
High K ⁺ + low Na	700 ± 50	6 ± 4	657 ± 14	63 ± 7	10.1
Veratridine $5 \cdot 10^{-6}$ M	629 ± 31	2 ± 2	—	—	—

Antagonism of the evoked release by uptake inhibitors

In a series of experiments the antagonism of the evoked release by various compounds was examined. To avoid the initial spontaneous release the vasa were pre-washed for 10 min at 37° . The release of radioactivity during a further 10 min period was measured. Imipramine methiodide was used besides desipramine as an inhibitor of the membrane NA transport since it as a quaternary ammonium derivative can be expected to preferably act on the outside of the membranes which minimizes intraneuronal interactions. Bethoxycaïne was used as a potent membrane stabilizing agent which has weak inhibitory action on the noradrenaline transport (Ross and Kelder 1976 b).

Imipramine methiodide antagonized the release of radioactivity evoked by (+)-amphetamine ($5 \cdot 10^{-6}$ M) and low external Na (26 mM) in reserpinized (Fig. 4) and normal vasa (Fig. 4). The release by veratridine ($5 \cdot 10^{-6}$ M) was only partially (15%) antagonized in the normal tissue but almost completely antagonized by imipramine methiodide in the reserpinized vasa. These findings agree with those previously obtained with desipramine (Ross and Kelder 1976 b). The release evoked by high external K (75 mM), low external Na (26 mM) and high K (117 mM) low Na (26 mM) was not apparently antagonized by imipramine methiodide (Fig. 4 and 5). Since high concentrations of imipramine methiodide itself caused significant release particularly in the reserpinized tissue, a partial antagonism is possible, if additive effects are assumed.

Desipramine had similar effects but was considerably more potent than imipramine methiodide (Table II) which is in accord with their potencies as uptake inhibitors (K_{1/2} in the rat vas deferens was $1 \cdot 10^{-6}$ M for desipramine and $2.3 \cdot 10^{-6}$ M for imipramine methiodide). Large concentrations of desipramine antagonized the release of radioactivity evoked by high K⁺ + low Na in the normal and reserpinized tissues (Fig. 6). These concentrations were 100 times larger than those antagonizing the veratridine evoked release in reserpinized vasa but equal to those effective against veratridine in normal tissue. As shown in Table II the two uptake inhibitors antagonized the release evoked by (+)-amphetamine, low Na and veratridine in reserpinized vasa at the same concentrations as they inhibited the accumulation of ^3H NA.

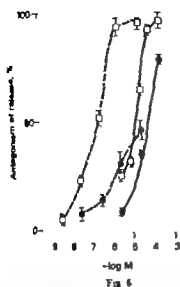


Fig. 6

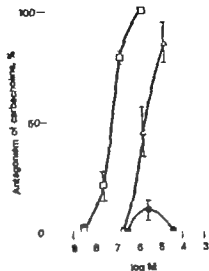


Fig. 7

6. Antagonism by various concentrations of desipramine on the release of ^3H -NA evoked by veratridine 10^{-6} M (□) and high K (117 mM) + low Na (26 mM) (●) in activated (boiled) and reserpined (not via defences). The incubation time was 20 min in the veratridine experiments and 10 min now with high K + low Na. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

7. Dose response curves of the antagonism by atropine (□), isoproprenaline methiodide (Δ) and desipramine (●) of the carbacholine chloride induced reduction of the release of ^3H MA evoked by high K (117) + low Na (26 mM) in the not via defences in vivo. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

The effect of carbacholine chloride was antagonized by atropine ($\text{IC}_{50} = 6 \cdot 10^{-6}$ M) by isoprenaline methiodide ($\text{IC}_{50} = 2 \cdot 10^{-6}$ M) but desipramine had poor antagonizing effect (Fig. 7).

The release evoked by high (hypertonic) K (75 mM) was not antagonized by carbacholine chloride neither at 10 nor at 5 min's incubation. The release evoked by veratridine was slightly (17%) antagonized by carbacholine (Table V). Atropine counteracted carbacho-

TABLE III. Effect of benhexycaine on the release of ^3H NA evoked by various agents. The concentration of benhexycaine was $2.5 \cdot 10^{-6}$ M. Each value is the mean \pm S.E. of 4 determinations.

	Δ Release, \pm S.E.			
	Normal tissue		Reserpined tissue	
	Control	Benhexycaine	Control	Benhexycaine
Amphetamine, $5 \cdot 10^{-6}$ M	7.6 \pm 0.9	9.8 \pm 1.9	31.0 \pm 1.6	30.0 \pm 1.3
Veratridine $5 \cdot 10^{-6}$ M	32.7 \pm 0.9	1.8 \pm 0.7***	34.6 \pm 1.7	0 \pm 0.4
low Na 26 mM	7.6 \pm 1.2	8.9 \pm 0.7	15.2 \pm 1.9	17.5 \pm 2.2
high K 75 mM	40 \pm 0.4	6.6 \pm 1.5	14.3 \pm 1.7	8.6 \pm 1.0
high K 75 mM + low Na 26 mM	37.5 \pm 0.9	33.6 \pm 0.7*	56.7 \pm 1.7	54.9 \pm 0.9

* $p < 0.05$ ** $p < 0.001$ (t-test).

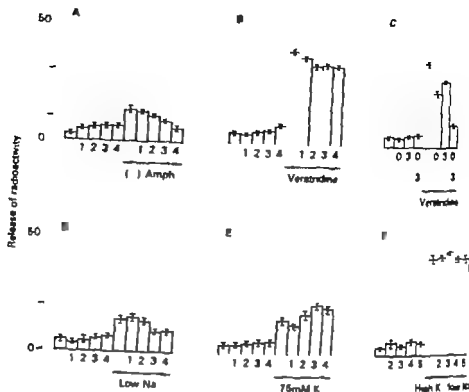


Fig. 5. Antagonism by imipramine methiodide of the release of ^3H -NA from normal rat vas deferens evoked by various means. The concentrations of the compounds were the same as in the experiments shown in Fig. 4.

Effect of carbacholine chloride Dubey *et al.* (1975) have shown that stimulation of muscarinic receptors on noradrenergic nerve terminals reduces the release of NA produced by high external concentration of K. We therefore examined, if the release of ^3H -NA from the vas deferens evoked by the various means examined was influenced by muscarinic agonists. In the non reserpinized tissue the release by high K + low Na was reduced maximally 36% by carbacholine chloride (Table V) and metacholine chloride. The concentration of these agents producing half of this maximal response was $5.5 \cdot 10^{-6}$ M for carbacholine chloride and $3.1 \cdot 10^{-6}$ M for metacholine chloride.

TABLE II. Effective concentrations of imipramine methiodide and desipramine in antagonizing the release of ^3H -NA from normal and reserpinized rat vas deferens *in vitro*. The values are interpolated from dose response curves of the type shown in Fig. 4 and 5.

	$\text{EC}_{50}, \mu\text{M}$			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
(+)-Amphetamine $5 \cdot 10^{-6}$ M	11.8	16.2	0.10	0.30
Low Na (26 mM)	14.7	8.8	—	0.35
Veratridine ($5 \cdot 10^{-6}$ M)	>245	8.8	11	0.28
High K (117 mM) + low Na ⁺ (26 mM)	>245	245	80	50

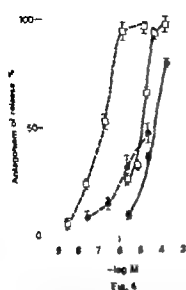


Fig. 4

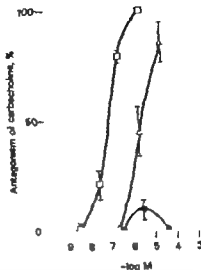


Fig. 7

6 Antagonism by various concentrations of desipramine on the release of ^3H MA evoked by veratridine 10^{-4} M (□) and high K (117 mM) low Na (26 mM) (●) in ascorbated (whole bars) and reserpinized (dashed bars) rat vas deferens. The incubation time is 20 min in the carbacholine experiments and 10 min here with high K. low Na. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

7 Dose response curves of the antagonism by atropine (□), isoprenaline methiodide (Δ) and desipramine (●) of the carbacholine chloride induced reduction of the release of ^3H MA evoked by high K (117 mM) low Na (26 mM) in the rat vas deferens *in vitro*. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

The effect of carbacholine chloride was antagonized by atropine ($\text{IC}_{50} = 6 \cdot 10^{-6}$ M) and by isoprenaline methiodide ($\text{IC}_{50} = 2 \cdot 10^{-6}$ M) but desipramine had poor antagonizing action (Fig. 7).

The release evoked by high (hypertonic) K (75 mM) was not antagonized by carbacholine chloride neither at 10 nor at 5 min's incubation. The release evoked by veratridine was slightly (17 %) antagonized by carbacholine (Table V). Atropine counteracted carbacholine.

III Effect of bethoxycaïne on the release of ^3H -MA evoked by various agents. The concentration of bethoxycaïne is $2 \cdot 10^{-6}$ M. Each value is the mean \pm S.E. of 4 determinations.

	Δ Release, \pm S.E.			
	Normal tissue		Reserpinized tissue	
	Control	Bethoxycaïne	Control	Bethoxycaïne
Veratridine $5 \cdot 10^{-5}$ M	76 ± 0.9	9.3 ± 1.9	31.0 ± 1.6	30.0 ± 1.3
Veratridine $5 \cdot 10^{-5}$ M	32.7 ± 0.9	$1.8 \pm 0.7^*$	34.6 ± 1.7	$0 \pm 0.4^{***}$
high K 26 mM	76 ± 1.2	8.9 ± 0.3	15.2 ± 1.9	17.5 ± 2.2
high K 75 mM	40 ± 0.4	4.6 ± 1.5	14.3 ± 1.7	8.6 ± 1.0
high K 75 mM 26 mM Na	37.5 ± 0.9	$13.6 \pm 0.7^*$	56.7 ± 1.7	54.9 ± 0.9

* $p < 0.05$, *** $p < 0.001$ (t-test).

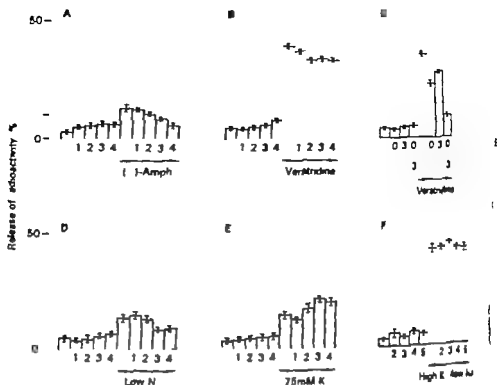


Fig. 5 Antagonism by imipramine methiodide of the release of ^3H NA from normal rat vas deferens evoked by various means. The concentrations of the compounds were the same as in the experiments shown in Fig. 4.

Effect of carbacholine chloride Dubey *et al* (1975) have shown that stimulation of muscarinic receptors on noradrenergic nerve terminals reduces the release of NA produced by high external concentration of K. We therefore examined if the release of ^3H -NA from the vas deferens evoked by the various means examined was influenced by muscarinic agonists. In the non reserpinized tissue the release by high K + low Na was reduced maximally 56% by carbacholine chloride (Table V) and metacholine chloride. The concentration of these agents producing half of this maximal response was 5.5×10^{-6} M for carbacholine chloride and 3.1×10^{-6} M for metacholine chloride.

TABLE II Effective concentrations of imipramine methiodide and desipramine in antagonizing the release of ^3H NA in normal and reserpinized rat vas deferens *in vitro*. The values are interpolated from dose response curves of the type shown in Fig. 4 and 5

	$\text{EC}_{50}, \mu\text{M}$			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
(+)-Amphetamine 5×10^{-6} M	11.8	16.2	0.10	0.30
Low Na (26 mM)	14.7	8.8	—	0.35
Veratridine (5×10^{-6} M)	>245	245	18	0.28
High K ⁺ (117 mM) + low Na ⁺ (26 mM)	>245	245	80	50



Fig. 6

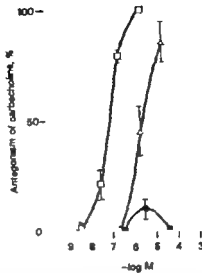


Fig. 7

Antagonism by various concentrations of desipramine on the release of ³H-NA evoked by veratridine 10^{-6} M (○) and high K⁺ (117 mM) low Na⁺ (26 mM) (●) as untreated (hole lines) and reserpinized (solid lines) rat vas deferens. The incubation time was 20 min in the veratridine experiments and 10 min in the high K⁺ + low Na⁺. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

Dose response curves of the antagonism by atropine (○), isoprenaline methiodide (Δ) and desipramine (●) of the carbacholine induced reduction of the release of ³H-NA evoked by high K⁺ (117 mM) low Na⁺ (26 mM) at the rat vas deferens *in vitro*. Each value is the mean \pm S.E. (vertical bars) of 4 determinations.

The effect of carbacholine chloride was antagonized by atropine ($IC_{50} = 6 \cdot 10^{-6}$ M) and by isoprenaline methiodide ($IC_{50} = 2 \cdot 10^{-6}$ M) but desipramine had poor antagonizing action (Fig. 7).

The release evoked by high (hypertonic) K⁺ (75 mM) was not antagonized by carbacholine chloride neither at 10 nor at 5 min incubation. The release evoked by veratridine was highly (17%) antagonized by carbacholine (Table V). Atropine counteracted carbacho-

linic effect of bethoxycaine on the release of ³H-NA evoked by various means. The concentration of bethoxycaine was $2.9 \cdot 10^{-6}$ M. Each value is the mean \pm S.E. of 4 determinations.

Incubation time	Δ Release \pm S.E.			
	Normal tissue		Reserpinized tissue	
	Control	Bethoxycaine	Control	Bethoxycaine
1 h + Amphetamine, $5 \cdot 10^{-6}$ M	7.6 ± 0.9	9.8 ± 1.9	31.0 ± 1.6	30.0 ± 1.3
Veratridine, $5 \cdot 10^{-6}$ M	32.7 ± 0.9	$1.8 \pm 0.7^{***}$	34.6 ± 1.7	$0 \pm 0.4^{**}$
Low Na ⁺ 26 mM	7.6 ± 1.2	8.9 ± 0.3	15.2 ± 1.9	17.5 ± 2.2
High K ⁺ 75 mM	4.0 ± 0.4	6.6 ± 1.5	14.3 ± 1.7	8.6 ± 1.0
7 M Na ⁺ 26 mM Na ⁺	37.5 ± 0.9	$33.6 \pm 0.7^*$	36.7 ± 1.7	34.9 ± 0.9

* $p < 0.05$, ** $p < 0.001$ (t-test).

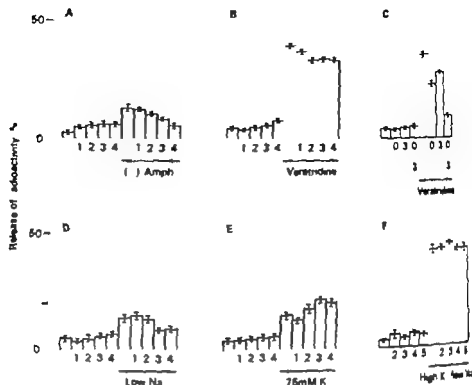


Fig. 5 Antagonism by Imipramine methiodide of the release of ^3H NA from normal rat vesicles evoked by various means. The concentrations of the compounds were the same as in the experiment shown in Fig. 4.

Effect of carbacholine chloride Dubey *et al.* (1975) have shown that stimulation of muscarinic receptors on noradrenergic nerve terminals reduces the release of NA produced by high external concentration of K^+ . We therefore examined if the release of ^3H -NA by the vas deferens evoked by the various means examined was influenced by muscarinic agonists. In the non-reserpinized tissue the release by high K^+ + low Na^+ was reduced maximally 56% by carbacholine chloride (Table V) and metacholine chloride. The concentration of these agents producing half of this maximal response was $5.5 \cdot 10^{-5} \text{ M}$ for carbacholine chloride and $3.1 \cdot 10^{-5} \text{ M}$ for metacholine chloride.

TABLE II Effective concentrations of imipramine methiodide and desipramine in antagonizing the release of ^3H NA in normal and reserpinized rat vas deferens *in vitro*. The values are interpolated from dose response curves of the type shown in Fig. 4 and 5.

	EC_{50} , μM			
	Imipramine methiodide		Desipramine	
	Control	Reserpine	Control	Reserpine
(+)-Amphetamine $5 \times 10^{-6} \text{ M}$	11.8	16.2	0.10	0.90
Low Na^+ (26 mM)	14.7	8.8	—	0.35
Veratridine $(5 \times 10^{-6} \text{ M})$	> 245	8.8	18	0.28
High K^+ (117 mM) + low Na^+ (26 mM)	> 245	> 245	80	50

11. Effect of clonidine, phentolamine and morphine on the release of ³H NA evoked by high K in rat ves deferens. The release of ³H-NA during 10 min is determined. Each value is the mean \pm S.E. of 4 determinations.

Compound	Δ Release of ³ H-NA, \pm S.E.		
	Conc. M	High K (75 mM)	High K (117 mM) + low Na (26 mM)
—		6.8 \pm 0.7	37.7 \pm 0.9
Clonidine	3.7 \cdot 10 ⁻⁶	7.3 \pm 1.2	38.5 \pm 1.0
Clonidine	3.7 \cdot 10 ⁻⁶	7.4 \pm 0.5	40.2 \pm 2.4
—		7.6 \pm 1.0	37.0 \pm 1.0
Phentolamine	1 \cdot 10 ⁻⁵	8.5 \pm 0.8	35.1 \pm 0.7
Morphine	1 \cdot 10 ⁻⁵	—	38.7 \pm 1.4

Discussion

results obtained are in accordance with the view that the release of NA from peripheral sympathetic nerve terminals occurs by two mechanisms.

A Ca²⁺ dependent release which is evoked by depolarizing agents (high K concentration and veratridine). This release is probably identical with the exocytotic release of NA (Ugels 1968, Smith and Winkler 1977).

Ca²⁺ independent, temperature sensitive release evoked by low external Na (+)-tubocurarine and veratridine. Since this release is inhibited by inhibitors of the NA uptake probably a carrier-mediated transport of NA (Paton 1973, Ross and Keider 1976 b), release is easily demonstrated in reserpinized tissue in which the exocytotic release of NA is abolished. However the release induced by (+)-amphetamine, low external Na is a part of that evoked by veratridine obviously occurs with this mechanism also in normal tissue.

The exocytotic release evoked by high K concentration has several similarities with the impulse propagated release of NA (Thoa *et al.* 1975). Thus, the muscarinic receptor agonistic carbacholine counteracted the release evoked by high K + low Na which is in accord with the hypothesis that muscarinic receptors on the NA nerve terminals modulate release of NA probably by antagonizing the influx of Ca²⁺ induced by the depolarization of the nerve membranes (Dubey *et al.* 1975). Like atropine imipramine methiodide but desipramine antagonized the effect of carbacholine which indicates that imipramine methiodide has an anticholinergic effect.

The potent pre-junctional α -adrenoceptor stimulating agent clonidine antagonizes and α -blocking compound phentolamine enhances the impulse propagated release of ³H NA (Wakade 1977). However in our system these compounds did not influence the K⁺ evoked release of ³H-NA in normal ves deferens. This observation agrees with those recently reported by Wakade and Wakade (1977) and by Garcia *et al.* (1978) in slices of the cat spleen. The latter authors suggested that the adrenoceptors on the nerve terminals are not able to modulate the release of NA when the membranes are strongly and long-lastingly depolarized by high K⁺ concentrations.

In accordance with the studies of Wakade and Karyekar (1974) and Paton and Laurzon (1977) we found that the release of ³H-NA evoked by high K⁺ concentrations in the reser-

TABLE IV Ca^{2+} dependence of the release of ^3H NA evoked by various means. The Ca^{2+} -free medium contained 1 mM EGTA. The radioactivity released into the medium during 10 min was expressed in per cent of the radioactivity in the tissue at the start of the release period. Each value is the mean \pm S.E. of 4 determinations.

Releasing agent	+ Δ Release % \pm S.E.		Reserpinized tissue ^a	
	Normal tissue			
	Control	- Ca^{2+}	Control	Ca^{2+}
(+)-Amphetamine $5 \cdot 10^{-6}$ M	9.1 ± 0.7	9.0 ± 0.7	32.3 ± 1.4	32.6 ± 1.0
Low Na 26 mM	9.8 ± 0.4	8.8 ± 0.8	21.1 ± 2.0	19.4 ± 1.0
High K 75 mM	6.8 ± 1.5	1.0 ± 0.5	8.0 ± 2.2	13.3 ± 1.9
117 mM K^{+} + 26 mM Na	38.1 ± 1.3	14.5 ± 0.3	56.3 ± 1.8	$44.7 \pm 1.2^{**}$

$p < 0.01$ $p < 0.001$ (t-test).

Reserpine 5 mg/kg i.p. 18 hours before the experiments.

line also in this respect. The release produced by (+)-amphetamine or by low external Na^{+} was not influenced by carbacholine (Table V).

In the reserpinized tissue the release induced by high K⁺ + low Na⁺ was slightly ($p < 0.05$) but significantly reduced by carbacholine chloride (Table V). This effect of carbacholine was antagonized by atropine and imipramine methiodide. The release evoked by the other means was not influenced by carbacholine in the reserpinized tissue.

Effect of α -adrenoceptor stimulating and blocking agents The pre-junctional α -adrenoceptor stimulating compound clonidine and the α -blocking agent phentolamine failed to influence the release of ^3H NA by high K⁺ + low Na⁺ or by hypertonic K⁺ (75 mM) in the rat vas deferens (Table VI).

Morphine At the concentrations examined morphine did not change the release of ^3H NA induced by high K⁺ + low Na⁺ (Table VI).

TABLE V Effect of carbacholine chloride ($5 \cdot 10^{-6}$ M) on the release of ^3H NA in rat vas deferens released by various means. The release of ^3H NA during 10 min was determined. Each value is the mean \pm S.E. of 4 determinations.

	+ Δ Release of ^3H NA, % \pm S.E.		Reserpinized tissue	
	Normal tissue			
	Control	Carbacholine	Control	Carbacholine
(+)-Amphetamine, $5 \cdot 10^{-6}$ M	6.2 ± 1.9	11.0 ± 1.1	31.0 ± 1.6	29.6 ± 2.1
Low Na (26 mM)	9.0 ± 1.0	8.8 ± 1.4	15.2 ± 1.9	11.6 ± 0.8
High K (117 mM) + low Na (26 mM)	35.2 ± 0.6	18.2 ± 0.8	49.6 ± 3.3	38.9 ± 3.4
Veratridine $5 \cdot 10^{-6}$ M	32.5 ± 1.4	27.1 ± 0.9	32.5 ± 1.4	33.3 ± 1.9

$p < 0.01$ (t-test).

7. D. M. Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit aorta. *Br. J. Pharmacol.* 1971, 49 614-627.
8. D. M. and P. A. LAZZO, On the reduced retention of extravascular noradrenaline in rabbit aorta induced by potassium. *Europ. J. Pharmacol.* 1977 43 27-32.
9. S. B. Structural requirements for uptake into catecholamine neurones. I. *The mechanisms of neuronal extraneuronal transport of catecholamines*, ed. by D. M. Paton, 1976. 67-93 Raven Press, New York.
10. S. B. and T. GONZOVY, On the mechanism of the accumulation of H-bretylium in peripheral sympathetic nerves. *Arch. Pharmacol.* 1973. 288 283-293.
11. S. B. and D. KILDES, Active transport of ³H-bretylium in the rat vas deferens in vitro. *Acta physiol.* 1976 a. 87 209-211.
12. S. B. and D. KILDES, Effect of veratridine on the fluxes of ³H-noradrenaline and ³H-bretylium in vas deferens in vitro. *Arch. Pharmacol.* 1976 b. 293 183-189.
13. S. B. and D. KILDES, Evoked efflux of H-bretylium by sympathomimetic esters from the rat vas deferens in vitro. *Acta pharmacol. (Kbh.)* 1977 40. 87-96.
14. S. B. and A. L. RYTH, Effect of (+)-amphetamine on the retention of ³H-catecholamines in slices of normal and reserpined rat brain and heart. *Acta pharmacol. (Kbh.)* 1978. 47 165-173.
15. A. A. and H. WINKLER, Fluorescent mechanisms in the release of catecholamines. In *Catecholamines*, ed. by H. Blaschke and E. Mutscholl, 1972. 538-617 Springer Verlag, Berlin.
16. K. Regulation of noradrenaline release by presynaptic receptor systems. *Rev. Physiol. Biochem. Pharmacol.* 1977 77 1-124. Springer Verlag, Berlin.
17. K. B. G. F. WOOTE, J. AXELSON and I. J. KOPPE, On the mechanism of release of norepinephrine in sympathetic nerves induced by depolarizing agents and sympathomimetic drugs. *Mol. Pharmacol.* 1975 11 15-18.
18. A. R. and S. M. KILPHEAR, Calcium-independent release of ⁴⁵Ca-norepinephrine from reserpine-treated guinea-pig vas deferens and seminal vesicle. *J. Pharmacol. exp. Ther.* 1974. 190 451-458.
19. A. R. and WAKABE, T. Factors controlling the output of norepinephrine from the rat vas deferens. *Pl. Proc.* 1977 36. 327.
20. MALMEQUIST, H. The estimation of total (free + conjugated) catecholamines and some catecholamine metabolites in human urine. In: *Methods of Biochemical Analysis*, Ed. by D. Gluck. 1968. 16. 293-326. Science Publishers John Wiley & Sons, New York.

pinized tissue is mainly independent on the presence of Ca^{++} and is accordingly antagonized by muscarinic agents. The slight reduction of the release evoked by high Na^+ observed when Ca^{++} was omitted may be due to a rest of vesicular ^3H -NA release by exocytosis. The release by high K^+ concentrations differed also from that induced by other means examined in that it was rather resistant against uptake inhibitors. Ten times higher concentrations of desipramine was necessary to antagonize this release, at the same concentrations which caused membrane stabilizing effect in the vessel experiment in normal tissue. However, it seems unlikely that the antagonism was produced by this mechanism, since bethoxycaine had no effect in membrane stabilizing concentrations. A possible explanation is that the release by high K^+ in the reserpinized vessel occurs by the same carrier mediated mechanism as that evoked by the other agents but that the effect of the uptake inhibitors are strongly reduced by high K^+ concentrations.

The biphasic dose response curve of (+)-amphetamine with an optimal releasing effect at about $1 \cdot 10^{-6}$ M is similar to that previously observed for the release of ^3H -bretylium in the rat vas deferens (Ross and Kelder 1976 a, 1977) and for the release of ^3H -NA in the rat heart atrium (Ross and Renyi 1978). The reason for the decrease at high concentrations of (+)-amphetamine is unclear. We have previously suggested the (+)-amphetamine at these concentrations inhibits the membrane transport mechanism (Ross and Kelder 1977) but might alternatively be due to other unidentified membrane effects.

It can be questioned as to whether the carrier-mediated release of NA has any physiological or pharmacological importance under *in vivo* conditions. It seems at least possible that the effect of indirectly acting sympathomimetic amines occurs by this release mechanism, although it is yet unclear. If the uptake inhibitors block the uptake of these amines into the nerve terminals or antagonize the carrier-mediated release of the transmitter (Ross 1976). At the depolarized state of the neuronal membranes during nerve activity the carrier-mediated release is probably of little if any importance, since the concentration of the amine in the neuroplasma is normally low. However, it appears likely that the re-uptake of the transmitter is diminished or abolished when the membranes are depolarized. At high stimulation frequency this type of release may become significant.

The present study shows that the release of ^3H NA in reserpinized vas deferens is very similar to that of ^3H bretylium in the normal (non-reserpinized) vas deferens (Ross and Kelder 1976 a, b) 1977). It is therefore likely that ^3H bretylium utilizes the NA membrane transport mechanism. Since bretylium is not released by the Ca^{++} dependent exocytotic release and passes the neuronal membrane by passive diffusion much slower than does NA and is not isotopically diluted in the tissue. ^3H bretylium appears to be a good model compound in studies of the membranal NA transport system.

References

- DOUGLAS, W. W. Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. The first Gaddum Memorial Lecture. *Brit. J. Pharmacol.* 1968 34 451-474.
- DURRY, M. P. E., MUSCHOLL, A. and FEJTER, Muscarinic inhibition of potassium-induced noradrenaline release and its dependence on the calcium concentration. *Arch. Pharmacol.* 1975 291 1-15.
- GARCIA, A. G. S., M. KIRPEKAR and R. PASCUAL, Release of noradrenaline from cat spleen slices by pressure. *Brit. J. Pharmacol.* 1978 62 207-211.

to decide whether membrane fusions and histamine release occur when isolated intact mast cell granules, surrounded by normal perigranular membranes, are incubated in Ca^{++} releasing solutions.

Methods

Isolation of intact mast cell granules

Male Sprague-Dawley rats (350–450 g) are used in each of the 12 expts. performed. The rats are anaesthetized under light ether anaesthesia by cutting the carotids and a small incision was made along the midline of the abdomen. The peritoneal cavity was washed with 9 ml of salt solution (NaCl 145 mM, KCl 4 mM, CaCl_2 0.9 mM) buffered with 10 mM v/v Sörensen phosphate buffer ($\text{Na}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ 10 mM), to which 1 mg of bovine serum albumin/ml was added. The peritoneal cavity was washed as an open incision. The mast cell suspension was layered onto discontinuous 30–40% Ficoll gradient gradients were centrifuged (350 g, 10 min) and the mast cells were collected from the diffuse band between the 30 and 40% Ficoll layers and from the bottom of the 40% Ficoll layer. The cells were washed twice in the above buffered salt solution containing bovine serum albumin. By this method, originally developed by Uvnäs and Thon (1959), cell suspension was obtained containing more 95% mast cells.

Mast cells were suspended in 31 ml ice-cold 0.34 M sucrose buffered with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), tissue culture buffer without metal binding capacity (M9 Edwards *et al.* 1974). The pH was adjusted to 7.0 with 1.0 M NaOH (Na⁺ concentration 50 mM). 5 ml aliquots cooled in ice are isolated in each of the 12 expts. with MSE 100 W ultrasonic separator set at 4 μm amplitude for 5 using probe with diameter of 10 mm. The isolated granules were centrifuged (350 g, 15 min, 4°C) and the granule-containing supernatants, containing about 60 intact granules, were then centrifuged (3 000 g, 20 min, 4°C).

Incubation procedure

Granule-containing pellets were incubated for 45 min at 25°C in 240 μl 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0. To this incubation medium was added either CaCl_2 (10 mM, 1 mM, 100 μM , 1 μM), MgCl_2 (10 mM) or NaCl (10 mM) as control. In another 4 expts. lower concentrations of MgCl_2 (1 mM, 100 μM , 10 μM) were used.

Isolation of granules and electron microscopy

After completing the incubation procedure half of each granule-containing suspension was taken for electron microscopy (Shore *et al.* 1979) as modified by Bergendorff and Uvnäs (1972) and protein (Lowry *et al.* 1951) after previous centrifugation and washing in 0.15 M NaCl buffered with Sörensen phosphate buffer (H_2PO_4 , KH_2PO_4 47 mM), pH 7.0, to release any histamine remaining in the "changed" granules. The histamine in the granule-containing suspension is then located solely in intact rat mast cell granules covered by perigranular membrane. The other half of the granule suspension (120 μl) was processed for electron microscopy. The granules were fixed with 60 μl 10% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3, for 20 min at 22°C. The suspension was then cooled down in an ice-bath for 5–10 min and adding 300 μl ice-cold 1.5% OsO_4 in 0.1 M cacodylate buffer. After fixation and centrifugation for 15 min, the fraction was contained in new 1.5% OsO_4 in the same buffer (35 min). The granule was obtained after centrifugation and washing twice in 0.1 M cacodylate buffer pH 5.0, was stained with 0.1% uranyl acetate (pH 5.0, 22°C, 30 min). After washing in 0.1 M acetate buffer pH 5.0, granule pellet was suspended in freshly prepared 2 μg (60°C) before being dehydrated in an acetone series, soaked in propylene oxide and finally embedded in Spurr low-viscosity embedding media. Sections for electron microscopy were cut on Reichert ultramicrotome OM U3 and stained with uranyl acetate and lead citrate before examination in Philips EM 301 electron microscope at 80 kV. X-ray sections for light microscopy are stained with solution of toluidine blue and azur A containing 5% glucose.

Materials

Bovine serum albumin (AB Lab, Stockholm, Sweden), Ficoll (AB Pharmacia, Uppsala, Sweden), HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, Calbiochem, Los Angeles, California, USA), glutaraldehyde 2.5% was specially purified for electron microscopy and Spurr low-viscosity embedding media (A. L. Laboratories, Reading, England) were used. All other substances are obtained from the usual commercial sources.

The influence of calcium on morphology and histamine content of isolated intact rat mast cell granules

By

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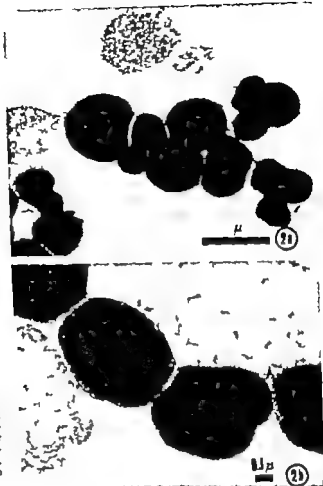
Abstract

ANDERSON P., P. RÖHLICH and B. UVNÄS. *The influence of calcium on morphology and histamine content of isolated intact rat mast cell granules.* Acta physiol. scand 1979 105: 350-358.

Histamine is released by "sequential exocytosis" in mast cells. The exocytosis involves fusion of the plasma membrane with the perigranular membrane and further fusions of adjacent perigranular membranes. To study a possible direct effect of Ca^{2+} on granule membrane fusions, mast cell granule suspensions were prepared from solicated rat mast cells. With the solication method used, more than 60% of the granules obtained were found to be homogeneous, electron dense and surrounded by a perigranular membrane, as observed in the electron microscope. These granules correspond to normal, histamine-containing granules found in untreated mast cells and are therefore named "intact" granules. The other granules were not less electron dense but without a perigranular membrane. These "changed" granules are formed during the histamine release process. Aliquots of the granule suspension were incubated in 0.34 M sucrose buffer with 10 mM HEPES, pH 7.0, containing different concentrations of CaCl_2 , MgCl_2 (10 mM, 1 mM, 0.1 mM, 10 μ M) or NaCl (10 mM). Only with the highest concentration (10 mM) of Ca^{2+} or Mg^{2+} was it possible to visualize an apposition of the perigranular membranes of "intact" normal granules. No characteristic lipid bilayer membrane structures could be observed at the place of membrane contact. Thus, at least no signs of membrane fusions. The histamine content was lower in the suspensions incubated with low concentrations of these ions or with 10 mM NaCl . Ca^{2+} and Mg^{2+} in high concentrations seemed to stabilize the perigranular membranes instead of initiating histamine release. Therefore, changes in the Ca^{2+} -to-concentration per se do not explain the membrane fusions seen in mast cells during "sequential exocytosis".

When sensitized mast cells are challenged with antigen (Anderson *et al.* 1973) or when normal mast cells are treated with, for example, compound 48/80 (Röhllich *et al.* 1977), histamine is released by sequential exocytosis. Sequential exocytosis (Röhllich *et al.* 1977) involves fusion of the plasma membrane with the perigranular membrane and further fusions between adjacent perigranular membranes. The granules involved in the fusion process alter from normal homogeneous, electron-dense granules to "changed" granules with a swollen, less electron-dense, reticular net-like appearance in the electron microscope.

Since the histamine release process is known to be Ca^{2+} -dependent (Mongar and Sjöberg 1958, Mota 1959, Mota and Iachil 1960, Chakravarty 1960, Högberg and Uvnäs 1960, Foreman and Mongar 1972, Douglas and Ueda 1973) it was considered of interest to



2. Mast cell granules incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing 10 mM CaCl_2 . The granules (with perigranular diaphragms) formed either aggregates or long chains of granules. Magnification: 217,000. 2b (a)

variable planes of sectioning (Fig. 3 a). The adhering membranes often also showed a wave-like pattern (Fig. 3 b). At the highest magnifications used, a 5-layered structure was observed, here the membranes were in contact (Fig. 3 a). A fusion of the membranes, a thinning of membrane layers from this pentalamellar appearance of the membrane contact, yielding a trilaminar diaphragm, or further elimination of individual layers along to a single-layered diaphragm (pore), was never observed in our material, when 10 mM CaCl_2 was used as the incubation medium.

The mixed granule suspensions were also incubated in 10 mM MgCl_2 in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0. The ultrastructural appearance was similar to that when 10 mM CaCl_2 was used. The intact granules again formed aggregates, often in the form of long chains. The perigranular membranes in contact either showed a wave-like pattern or were parallel to each other for a long distance, forming a 5-layered substructure (Fig. 3 c). Fusion of the membranes did not occur.



Fig. 1 Mast cell granules incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, and 10 mM NaCl (a) Homogeneous, electron-dense intact normal granules and swollen, less electron-dense "changed" granules are seen. No contact between the granules is observed. (b) Two intact granules adhere to each other. A perigranular membrane can be seen around each of the granules. The perigranular membranes are not in contact with each other (Magnification: 1a 14 000 \times 1b 57 500 \times)

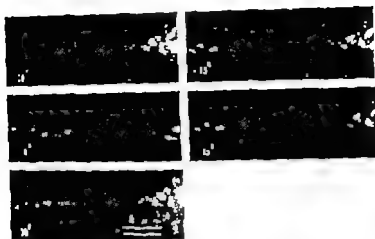
Results

Electron microscopy

The mast cell granule suspensions, obtained by our technique using ultrasonic disintegration of mast cells and differential centrifugations, were uncontaminated by other cell organelles. Between 60 and 70 % of the granules had a normal appearance (intact granules). They were surrounded by a perigranular membrane. These granules were homogeneous, electron-dense and thus similar to those found in untreated mast cells. The other granule type was less electron-dense with a swollen, reticular net-like appearance and thus corresponded to the "changed" granules found in mast cells during the histamine release process.

When mast cell granules were incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0 containing 10 mM NaCl (control medium), the granules were seen to have the same appearance as described above (Fig. 1 a) and in earlier experiments using a medium consisting of only 0.34 M sucrose (Anderson *et al* 1974). At these magnifications it was evident that the granules were lying separately and not in contact with each other (Fig. 1 a, b). The electron dense granules were surrounded by a membrane (Fig. 1 b), which was missing around the "changed" granules.

A completely different picture was observed when the mast cell granule suspensions were incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing 10 mM CaCl₂ (Fig. 2 and 3). Most of the normal intact granules were adhering to similar homogeneous granules, forming aggregates or long chains of granules (Fig. 2 a, b). The adhering membranes often maximized their area of contact, leading to distortions of the spherical granules. A "changed" granule could sometimes also be associated with the aggregates of granules. At higher magnifications it was observed that the membranes in contact were often oriented parallel to each other (Fig. 3 a) and adhering to each other at the contact site. The trilaminar structure of the individual membranes could be observed



4 Mast cell granules incubated as in Fig. 2. By tilting the specimen in goniometer stage the continuity of the separate membranes can be seen ($\pm 0^\circ$) (Magnification: 102,000 \times)

incubated in 0.14 M sucrose buffered with 10 mM HEPES (pH 7.0) containing 10 mM CaCl_2 and 0.01 M MgCl_2 was higher (0.127 and 0.110 respectively) than in the granule suspensions incubated with 10 mM NaCl (0.089). The difference is statistically significant at the 1% level. When the granule suspensions were incubated in lower concentrations of CaCl_2 and MgCl_2 , on the other hand, the histamine content did not differ from the control incubations.

Discussion

In exocytosis, the secretory products stored in membrane-limited vesicles or granules are released to the extracellular space without preliminary discharge into the cytoplasm and without concomitant loss of cytoplasmic constituents. It has been shown that an intracellular increase of calcium ions is essential for exocytosis in many cell types. However, it is not known how Ca^{2+} induces the fusion process of exocytosis.

We have shown earlier (Rohlich *et al.* 1971, Anderson *et al.* 1973, Anderson 1975), that mast cells are an excellent model for studying exocytosis. In mast cells the exocytosis is

Table 1 The effect of Ca^{2+} and Mg^{2+} on the histamine content (histamine/protein ratio) of isolated intact mast cell granules

	Mean	S.D.
0.14 M CaCl_2	0.127	0.053
0.01 M CaCl_2	0.094	0.034
0.001 M CaCl_2	0.067	0.040
0.0001 M CaCl_2	0.064	0.043
0.01 M MgCl_2	0.110	0.049
0.001 M NaCl	0.089	0.030

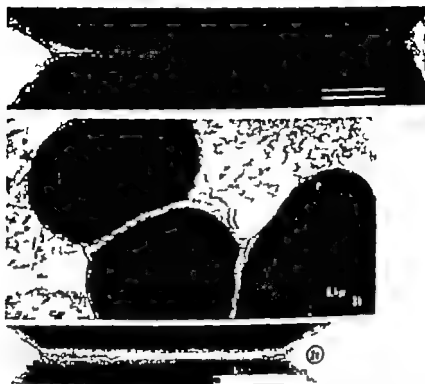


Fig. 3 Mast cell granules were incubated as in Fig. 2. (a) The perigranular membranes are oriented parallel to each other at the contact site. The intralaminar structure of each membrane can be observed at the left. (b) The opposed membranes often also showed a wavy pattern. (c) Mast cell granules incubated in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0, containing 10 mM $MgCl_2$. The layered structure is easily seen, where the membranes oppose each other. No fusion of the membranes occurs. (Magnification: 3a 165 000, 3b 48 000, 3c 165 000 \times)

Obliquely sectioned membranes often gave the impression of a fusion of the adjacent membranes. To exclude the possibility of an incorrect interpretation, we tilted some of our specimens in a goniometer stage of the electron microscope. In all of such tilted specimens we were able to follow the continuity of the separate membranes and ruled out a real fusion between the membranes in contact (Fig. 4).

When the mixed mast cell granules were incubated with 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0 containing lower concentrations of $CaCl_2$ (1 mM, 100 μM , 10 μM) or $MgCl_2$ (1 mM, 100 μM , 10 μM) the same morphology was found as in the control suspensions containing 10 mM NaCl in HEPES-buffered sucrose.

Histamine content

Histamine and protein were analyzed in the granule fractions after completing the incubations in 0.34 M sucrose buffered with 10 mM HEPES, pH 7.0 containing different concentrations of $CaCl_2$, $MgCl_2$ or NaCl. The histamine contents of the fractions were expressed as the histamine/protein ratio (Table 1). This ratio also reflects the proportion of "intact" granules in the fractions, since only "intact" granules surrounded by a perigranular membrane retain histamine, when washed in isotonic sodium chloride solution (Uvnäs *et al.* 1970, Anderson *et al.* 1974). The histamine

granule fractions

should be cautious when interpreting electron micrographs of subcellular fractions, as brines may become more fragile during *in vitro* preparation procedures, leading to external membrane ruptures.

Exocytosis in mast cells is dependent not only on Ca^{++} but also on energy (Hogberg and Nilsson 1957; Peterson 1974). It is not yet known, however, where energy is utilized during the release process. Therefore it is conceivable that the intracellular increase in the calcium concentration is just the initial step in a chain of events leading to complete fusion of the brines. After stimulation of mast cells, calcium may thus act as a secondary messenger and activate some mechanism that requires metabolic energy. If this is the case, exocytosis would be expected in an *in vitro* system. A candidate for such a calcium and energy-requiring system could be a contractile acto-myosin system (microfilaments), the existence of which in exocytosis is still debated (Röhlich 1975).

The adhesive property of calcium and magnesium ions in our experiments is probably specific for the release process, but may be explained by the divalent character of the ions.

Thus, it is known that these divalent ions play an important role in adhesions of membranes and cells in general. The adhesive effect can probably be explained by the fact that the ions act as bivalent bridges between negatively charged groups on the membrane (cf. Banks 1966). Our results showed a significantly higher histamine content in vesicle preparations treated with high Ca^{++} or Mg^{++} concentrations (10 mM). This finding may be due to a stabilizing effect of these ions on the perigranular membrane, instead of inducing a fusion process between them.

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References

- BRUN, P. Release of histamine from rat mast cells. An electron microscopic study. Thesis, Stockholm 1973.
- BRUN, P. & A. SÖDERBACH and B. URVIL, Sequential exocytosis of storage granules during antigen-induced histamine release from sensitized rat mast cells *in vitro*. An electron microscopic study. *Acta physiol. scand.* 1973, **88**, 359-372.
- BRUN, P., P. RÖHLICH, S. A. SÖDERBACH and B. URVIL, Morphology and storage properties of rat mast cell granules isolated by different methods. *Acta physiol. scand.* 1974, **91**, 145-153.
- BRUN, P. An interaction between chromaffin granules and calcium ions. *Biochem. J.* 1966, **101**, 18 c.
- BRUN, P. and B. URVIL, Storage of 5-hydroxytryptamine in rat mast cells. Evidence for an interaction with carboxyl groups in granule heparin-protein complex. *Acta physiol. scand.* 1972, **84**, 320-331.
- ALLAN, M. T. The mechanism of histamine release in anaphylactic reaction in guinea pig and rat. *Acta physiol. scand.* 1960, **48**, 146-166.
- BRUN, P. and M. GRÄTZL, Calcium-induced fusion of isolated secretory vesicles from mast of Langerhans. *Endocrinology* 1976, **117**(2), 344-355.
- BRUN, P. & A. SÖDERBACH, Involvement of calcium in exocytosis and the exocytosis-vesiculation sequence. *Biochem. Soc. J.* 1974, **139**, 1-28.
- BRUN, P. & A. SÖDERBACH, Exocytosis and the exocytosis-vesiculation sequence: with special reference to neuroendocrine, chromaffin and mast cells, calcium and calcium ionophores. In *Secretory mechanisms of neuroendocrine glands*. Ed. THOREN, K. and O. PETTERSSON. Munksgaard, Copenhagen 1974, pp. 116-129.
- BRUN, P. & B. URVIL, Mast cell secretion (histamine release) induced by 48-80 calcium-dependent exocytosis inhibited strongly by cytochalasin only when glycolysis is rate-limiting. *J. Physiol. (Lond.)* 1971, **231**, 97-99.

sequential in space and time ("sequential exocytosis"). This means that not all the granules move to the plasma membrane. Instead there is a chain-like fusion of the membrane-limited granules. Sequential exocytosis in mast cells has been reported by other authors (Lagunoff 1972, 1973; Douglas 1974 a, b).

As in other cell types, Ca^{2+} is necessary for the secretory process in mast cells (for details see Introduction). However, it is still unclear how calcium is involved in membrane fusions during exocytosis. One of the possibilities is a direct calcium effect on the membranes, leading to fusions of adjacent perigranular membranes.

To investigate the possibility of a direct effect of calcium on the membranes we used a pure mast cell granule fraction consisting of a high percentage of normal, non-membrane limited granules. These suspensions were assumed to be a suitable model for studying membrane fusions, since the perigranular membrane is able to fuse during exocytosis not only with plasma membranes but also with other perigranular membranes.

As seen from the results, only when using the highest concentration of CaCl_2 (10 mM) we were able to observe an effect of calcium on the perigranular membranes. Instead of fusion, there was a close apposition of perigranular membranes, resulting in a possible appearance of the membranes in contact. Even at places where it was difficult to observe the individual membranes in contact and which could be taken as a place of fusion, we could show by tilting the specimens in a goniometer stage, that the apposed membranes were intact and distinct. This apposition of the membranes does not seem to be specific for calcium, since magnesium in the same concentration had a similar effect. In fact, it has been shown that magnesium was not effective in inducing exocytosis when injected into mast cells (Kanno *et al.* 1973). In addition, the calcium concentration which induces adhesion of perigranular membranes in our experiments is probably well above the equal intracellular calcium level during stimulation in other cell types.

Experiments on the effects of calcium on isolated secretory vesicles from other cell types are contradictory. Edwards *et al.* (1974) described apposition and rupture of perigranular membranes in isolated chromaffin granules suspended in high concentrations of calcium or magnesium (5–10 mM). Similarly, Dahl and Gratzl (1976) observed calcium-induced fusions of isolated secretory vesicles from the islets of Langerhans. Using freeze-fracturing to detect membrane fusions, they found the effective calcium concentration to be much lower (*i.e.* 1 mM).

On the other hand, Schober and collaborators (1977) found an apposition and a post-fusional structure but no fusions at the places of membrane contact in isolated chromaffin granule fractions, when using 10 mM CaCl_2 . Palade was also unsuccessful in inducing *in vitro* fusion by calcium ions of membrane limited granules isolated from pancreatic acinar cells (personal communication 1976).

These contradictory results have various explanations. The experiments cited above differed from each other in biological material, isolation procedures, incubation media and electron microscopic techniques. Some of the prerequisites for exocytotic membrane fusions may be lacking in some of these *in vitro* systems (energy, enzymes, microfilaments, etc.). Moreover, one can not exclude the possibility that specific sites on the membranes are altered or completely lost during the preparation procedure. On the other hand,

Determination of 6-keto-prostaglandin $F_{1\alpha}$ in rabbit kidney and urine and its relation to sodium balance

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Abstract

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occurrence of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) as demonstrated in rabbit kidney medulla
cortex by mass-spectrometry. The post-mortem accumulation of 6-keto-PGF $_{1\alpha}$ was studied by mass-
spectrometry in regions of the rabbit kidney using (3,3,4,4- 4H) 6-keto-PGF $_{1\alpha}$ as an internal standard.
cortex contained $1.4 \pm 0.3 \mu g/g$, the medulla $2.1 \pm 0.6 \mu g/g$ and the papilla 3.7 ± 0.7 (S.E.) $\mu g/g$. The
medulla of 6-keto-PGF $_{1\alpha}$ is about 5 fold higher in the cortex than reported for PGE $_2$ and PGF $_{2\alpha}$.
The accumulation of PGE $_2$ and PGF $_{2\alpha}$ demonstrates over 6-keto-PGF $_{1\alpha}$ in the medulla and the papilla. The
presence of 6-keto-PGF $_{1\alpha}$ in rabbit urine as demonstrated by mass-fragmentography. On low salt
introduced, female rabbits (a) excreted Na 0.09 ± 0.01 (S.E.) mmol/day 6-keto-PGF $_{1\alpha}$ $11.8 \pm$
 $1.2 \mu g/day$ and nonexcretory PGF $_{2\alpha}$ (PGF $_{2\alpha}$) $2.0 \pm 0.6 \mu g/day$. Two day treatment with acetylsalicylic
(100 mg/kg) reduced urinary excretion of 6-keto-PGF $_{1\alpha}$ and iPGF $_{2\alpha}$ by 71 and 83 % respectively
($p < 0.05$), but sodium excretion was unchanged. On the same diet supplemented with NaCl the
rats excreted Na 27.5 ± 3.4 mmol/day ($p < 0.05$), 6-keto-PGF $_{1\alpha}$ $13.3 \pm 4.6 \mu g/day$ ($p < 0.05$) and iPGF $_{2\alpha}$
 $0.70 \mu g/day$ ($p < 0.05$). 6-keto-PGF $_{1\alpha}$ is a major metabolic product of prostacyclin (PGI $_2$). The occurrence
of 6-keto-PGF $_{1\alpha}$ in kidney and urine indicates considerable synthesis of PGI $_2$ in the kidney. The data
urinary PG excretion indicate the increased synthesis of PGI $_2$ in contrast to PGF $_{2\alpha}$, is not influenced
diary excretion of NaCl.

with Acetylsalicylic acid, gas chromatography-mass spectrometry prostacyclin (PGI $_2$), prostaglan-
F $_{1\alpha}$ /PGF $_{2\alpha}$, radioimmunoassay rabbit kidney sodium deprivation

the kidney has a high capacity to synthesize prostaglandins (PGs) (Hamberg 1969). The
process is predominantly located to the renal medulla, which has a tenfold higher capacity
synthesize PGE $_2$ and PGF $_{2\alpha}$ than the cortex (Larsson and Ånggård 1973, Larsson and Ång-
gård 1976, Poog and Levine 1976). Studies on the renal PGs indicate, however, that they
be of physiological importance in both the renal medulla and in the renal cortex.
the medulla, PGs seem to oppose the action of the antidiuretic hormone (Grantham and
1968, Anderson *et al.* 1975). In the cortex, PGs might be of importance for renal

- EDWARDS, W. J. H. PHILLIPS and S. J. MORRIS, Structural changes in chromaffin granules induced by divalent cations. *Biochim. biophys. Acta* (Amst.) 1974 336 164-173.
- FOREMAN, J. C. and J. L. MONGAR, The role of the alkaline earth ions in anaphylactic histamine release. *J. Physiol. (Lond.)* 1972 244 753-769.
- HÖÖBERG, B. and B. UVNÄS, The mechanism of the disruption of mast cells produced by compound 48/80. *Acta physiol. scand.* 1957 41 345-369.
- HÖÖBERG, B. and B. UVNÄS, Further observations on the disruption of rat mesenteric mast cells by compound 48/80, antigen antibody reaction, lecithinase A and decylamine. *Acta physiol. scand.* 1964 91 133-145.
- KANNO, T., DE COCHRANE and W. W. DOUGLAS, Exocytosis (secretory granule extrusion) induced by contraction of calcium into mast cells. *Canad. J. Physiol. Pharmacol.* 1973 51 1001-1004.
- LAQUOFF, D. The mechanism of histamine release from mast cells. *Biochem. Pharmacol.* 1972 21 189-1896.
- LAQUOFF, D. Membrane fusion during mast cell secretion. *J. cell Biol.* 1973 37 252-258.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951 193 265-275.
- MONGAR, J. L. and H. O. SCHILD, The effect of calcium and pH on the anaphylactic reaction. *J. Physiol. (Lond.)* 1958 140 272-284.
- MOTA, I. Effect of antigen and octylamine on mast cells and histamine content of sensitized guinea pig tissues. *J. Physiol. (Lond.)* 1959 147 425-436.
- MOTA, I. and T. LIND, Inhibition of mast cell disruption and histamine release in rat anaphylaxis. Comparison with compound 48/80. *Brit. J. Pharmacol.* 1960 15 8-87.
- PETERSON, C., Role of energy metabolism in histamine release. A study on isolated rat mast cells. *J. Physiol. scand.* 1974, Suppl. 413.
- RÖHLICH, P., P. ANDERSON and B. UVNÄS, Electron microscope observations on compound 48/80-induced degranulation in rat mast cells. Evidence for sequential exocytosis of storage granules. *J. cell Biol.* 1975 51 465-483.
- RÖHLICH, P. Membrane-associated actin filaments in the cortical cytoplasm of the rat mast cell. *Exp. Cell Res.* 1975 93 293-298.
- SCOBIE, R. C., NITSCHE and U. RÖNN, Calcium-induced displacement of membrane-associated particles upon aggregation of chromaffin granules. *Science* 1977 195 495-497.
- SHIMMAN, C. Jr., Evaluation of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) as a tissue culture buffer. *Proc. Soc. exp. Biol. (N.Y.)* 1969 130 305-310.
- SHORE, P. A., A. BURKHALTER and V. H. COHEN, A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. exp. Ther.* 1959 127 184-186.
- UVNÄS, B. and I. L. THOM, Isolation of biologically intact mast cells. *Exp. Cell Res.* 1959 17 52-53.
- UVNÄS, B., C. H. ÅBERG and A. BERGENDORFF, Storage of histamine in mast cells. Evidence for the binding of histamine to protein carboxyls in the granule heparin-protein complex. *Acta physiol. scand.* 1970 98 Suppl. 336.

plates through the silicic acid layer. The references were localized by spraying the sides of the plates with phosphomolybdic acid followed by gentle heating. The zone of the chromatogram corresponding to reference (R) 6.52 was scraped off and eluted with 5% methanol in ethyl acetate (2 vol. 2). After evaporation, the samples are methylated by treatment with 0.2 ml 0.1 methoxyaniline HCl in acid overnight at room temperature. The samples are then evaporated to dryness and methylated in fresh ethereal solution of diazomethane. After evaporation trimethylsilyl ethers were prepared by adding the samples to 30 µl BSTFA for at least 1 h at room temperature. After evaporation the residues were dissolved in n-hexane (30 µl) and 5 µl was injected into the combined gas chromatography-mass spectrometer (GC-MS).

Analyses. Female, non-pregnant albino rabbits (6, 2.0–2.5 kg) were kept in separate metabolic cages. The cages were cleaned mechanically and rinsed with water daily. Urine was collected daily in 100 ml beakers placed with dry ice in thermos flasks in order to avoid degradation of PGs. The urine was collected immediately after voiding and was kept frozen until analysed.

Rabbits were first fed the low salt diet (Purina test diet "0.05% NaCl") and fed water supplemented with 0.5% very heavy water. Water was offered *ad libitum*. After an equilibrium period of two weeks, urine was collected for two days. The rabbits were then treated with acetylsalicylic acid (30 mg/kg 2 s.c.) for 10 days. Urine collection of PG system. The rabbits were then fed the basal diet (Purina test diet "Basal") and were supplemented with straw fibers (10–15 g hay) and 0.5% NaCl in water *ad libitum*. After one week equilibrium (two weeks after aspirin treatment) urine was collected for two days as described above. Urinary concentration of 6-keto-PGF_{1α} was analysed by semi-fragmatography. To 10 ml urine 100 µl of 0.1 M HCOOH and extracted twice with an equal volume of CHCl₃. To obtain phase separation the mixture was shaken at 1500 g for 15 min and after centrifugation. The CHCl₃ extracts were dried by filtering through 4 g Na₂SO₄ on filter paper and evaporated. The urine samples were then purified by thin layer chromatography and derivatized as described above.

Urine and urine samples were run on an LKB 2091 GC-MS which is equipped with multiple ion detector. A 1 m OV-17 GLC column (30 cm) was used and operated at 250°C. The energy of the electrons was 2.5 eV and the trap current was 100 µA. For the mass fragmentographic analysis the instrument was focused on m/z 602/396 (M⁺ 11) and 512/308 (M⁺ 121), but for routine analysis on the higher ions. The method for 6-keto-PGF_{1α} analysis is a modification of the one described by Pace-Asciak *et al.*

Urine and potassium in urine were measured by flame photometry using lithium as an internal standard. Urine was measured as unextracted urine by radioimmunoassay (RIA) as previously described (Oliver *et al.*). The assay has been validated for rabbit urine analysis by direct comparison with GC-MS analysis. Good agreement between the two methods were found (Davies *et al.* 1978). The values for PGF_{1α} and by RIA are referred to as acetylsalicylate-PGF_{1α} (aPGF_{1α}) in the text.

Student's *t*-test was used for statistical analyses and *p* < 0.05 considered significant. Data are expressed as S.E.

Results

spectrum of 6-keto-PGF_{1α}

Structure of the 6-keto-PGF_{1α} derivative for GC-MS analysis, 6-methoxymethyl-6-(15-trimethylsilyloxy-9-oxo-9H-fluoren-2-yl) ester 9-[[15-trimethylsilyloxy] ether] is shown in Fig. 1 top. A mass spectrum of 6-keto-PGF_{1α} is demonstrated in Fig. 1 A. The deuterated 6-keto-PGF_{1α} standard contained less than 1% of the proton form. The characteristic ions of high mass in the upper mass range were m/z 602 (M⁺ 31), 560 (M⁺ 71), 512 (M⁺ 121) and 460 (M⁺ 161).

The occurrence of 6-keto-PGF_{1α} in rabbit kidney medulla and cortex was demonstrated by identification of the major fragments. This is shown in Fig. 1 B by a mass spectrum of 6-keto-PGF_{1α} isolated from the renal medulla. A similar pattern was found in the renal cortex.

6-keto-PGF_{1α} standards were unstable in absolute ethanol or methanol. The order

blood flow distribution and for renin release (Larsson and Ånggård 1974, Larsson *et al* 1974, Weber *et al* 1976).

In 1976 Moncada, Vane and coworkers discovered that arterial walls can synthesize a novel PG like product from arachidonic acid. Its structure was elucidated and the compound was named prostacyclin PGI_2 (Johnson *et al* 1976). PGI_2 is unstable in aqueous solution, where it is hydrolysed to 6-keto-PGF. Since this is the only known pathway for 6-keto-PGF formation, the level of 6-keto-PGF $_{1\alpha}$ in a tissue might be used to estimate PGI_2 biosynthesis (Gibson 1977).

Pace Aslak and Rangaraj (1977) demonstrated that rat renal homogenates form 6-keto-PGF $_{1\alpha}$ from arachidonic acid. The physiological function of PGI_2 or 6-keto-PGF $_{1\alpha}$ in the kidney is unknown. Recent studies show that exogenous PGI_2 can influence renal haemodynamics. PGI_2 increases renal blood flow and stimulates renin release *in vitro* and *in vivo* (Katz *et al* 1978 a, Whorton *et al* 1977).

The present study describes the regional distribution of the endogenous formation of 6-keto-PGF $_{1\alpha}$ in the rabbit kidney. Since variations in sodium intake influence renal PG synthesis in rats (Tobian and O'Donnell 1976) and urinary excretion of PGE_2 and PGI_2 in rabbits (Weber *et al* 1977, Davila *et al* 1978), we also studied the urinary excretion of 6-keto-PGF $_{1\alpha}$ in rabbits on low and high salt intake.

Material and methods

Materials

All reagents were of analytical grade. PGF $_{1\alpha}$, 6-keto-PGF $_{1\alpha}$ and (3,3',4,4'-H $_4$) 6-keto-PGF $_{1\alpha}$ were gifts from the Upjohn Co. (Kalamazoo, USA) through the courtesy of Dr U. Årén. The 6-keto-PGF $_{1\alpha}$ standards were dissolved in ethyl acetate containing 5% methanol and stored at -20°C . The deuterated 6-keto-PGF $_{1\alpha}$ contained less than 1% of the protium form according to mass fragmentography and is suitable for chemical purity by thin layer chromatography. Methoxyamine HCl and BSTFA (N,O-bis(trimethylsilyl) trifluoroacetamide) were obtained from Pierce, Rockford, USA. The thin layer silica and plates were obtained from Merck (20 x 20 cm, 0.25 mm DC Ferrug-Platten Kieselgel 60). Acetylcholine and acetylcholinesterase were obtained from Boehringer-Mannheim. The PGF $_{1\alpha}$ antibodies were raised in our laboratory and characterized as previously described (Oliv *et al* 1978). The test diets, one basal diet (Lib 89) and one low salt diet with 0.03% NaCl but otherwise similar, were obtained from Ralston Purina Co., St. Louis, Indiana, USA.

Methods

Kidney analysis. Male white albino rabbits (2.5 kg) were kept on a standard pellet diet (Ammal Bördertillje, Sweden, containing 0.5% NaCl) and water *ad libitum*. The rabbits were killed by a blow to the head and exsanguinated. To standardize the procedure, the kidneys were removed 5 min after death and then immediately put into ice-cold 0.1 M potassium phosphate buffer pH 7.4. The following work was rapidly performed at 4°C . The cortex, medulla and papilla of each kidney were macroscopically dissected, weighed (0.4–1.5 g) and chopped into small pieces. The papillae from each pair of kidneys were put into 4 volumes of the phosphate buffer and 1.0 μg (3,3',4,4'-H $_4$) 6-keto-PGF $_{1\alpha}$ were added before homogenization in a glass tube with 5 strokes of a loose fitting Teflon pestle at 2500 r.p.m. The homogenate was centrifuged for 60 min at 90 000 g (Spinco L2 ultracentrifuge).

Proteins in the supernatant were precipitated by addition of 10 volumes of ethanol. The solution was rapidly filtered and evaporated almost to dryness. The residue was dissolved in 2 ml acetic acid, pH 3 with 0.1 M HCOOH and extracted with ethyl acetate (2 ml x 2). After evaporation, the extracts were purified by preparative thin layer chromatography (TLC) on silica acid TLC plates (Kieselgel 60, Merck) using trimethylpentane/acetic acid as solvent (10:17:20:100). Reference 6-keto-PGF $_{1\alpha}$ was put at the top of the plate. The references were separated from the samples by vertical lines drawn on both

plates through the silicic acid layer. The references were localized by spraying the sides of the plates with phosphomolybdic acid followed by gentle heating. The zone of the chromatogram corresponding to reference (Rf 0.32) was scraped off and eluted with 5 ml methanol in ethyl acetate (2 ml:2). After evaporation, the samples were methylated by treatment with 0.1 and 0.1 ml methoxyamine HCl solution overnight at room temperature. The samples were then evaporated to dryness and methylated with ethereal solution of diazomethane. After evaporation trimethylsilyl ethers were prepared by treating the samples with 50 µl BSTFA for at least 1 h at room temperature. After evaporation the residue was dissolved in hexane (50 µl) and 5 µl was injected into the combined gas chromatography-mass spectrometer (GC-MS).

Animals. Female, non-pregnant albino rabbits (±6, 2.0–2.5 kg) were kept in separate metabolic cages. The cages were cleaned mechanically and rinsed with water daily. Urine was collected daily in bottles, which were placed with dry ice in thermos flasks in order to avoid degradation of PGs. The urine was chilled immediately after voiding and was kept frozen until analyzed.

Rabbits were first fed the low salt diet (Purina test diet "0.05% NaCl") and *ad libitum* supplemented with 5 g hay daily. Water was offered *ad libitum*. After an equilibrium period of 10 weeks, urine was collected for two days. The animals were then treated with acetylsalicylic acid (30 mg/kg 2 ×) for 10 days as an inhibitor of PG synthesis. The rabbits were then fed the basal diet (i.e. Purina test diet "Basal") and supplemented with screw fibers (10–15 g hay) and 0.5% NaCl in water *ad libitum*. After one week equilibrium (two weeks after aspirin treatment) urine was collected for two days as described above. Every concentration of 6-keto-PGF_{1α} was analyzed by mass-fragmentography. To 10 ml urine in which was added 0.5 or 1.0 µg (3,3,4,4-²H₄) 6-keto-PGF_{1α}. The urine was acidified (pH 3) with 15–20 µl of 8 M HCOOH and extracted twice with an equal volume of CHCl₃ to obtain phase separation. The organic phase was dried by filtering through 4 g Na₂SO₄ on Whatman paper and evaporated. The urine samples were then purified by thin layer chromatography and derivatized as described above.

Urine and urine samples were run on an LKB 2091 GC-MS which was equipped with multiple ion detector. A 100 m OV 17 GLC column (80 cm) was used and operated at 250°C. The energy of the electrons was 25 eV and the trap current was 100 µA. For the mass fragmentography analysis the instrument was set to scan at m/e 602/598 (M-31) and 512/508 (M-121), but for routine analysis on the higher mass. The method for 6-keto-PGF_{1α} analysis is a modification of the one described by Pace-Asciak

and potassium in urine were measured by flame photometry using lithium as an internal standard. Urine was extracted with methanol and analyzed by radioimmunoassay (RIA) as previously described (Oliver *et al.*). The assay has been validated for rabbit urine analysis by direct comparison with GC-MS analysis and agreement between the two methods was found (Devila *et al.* 1978). The values for PGF_{1α} determined by RIA are referred to as immunoreactive PGF_{1α} (iPGF_{1α}) in the text.

Statistical tests used for statistical analysis and $p < 0.05$ considered significant. Data are expressed as mean ± SE.

Results

Spectrum of 6-keto-PGF_{1α}

The structure of the 6-keto-PGF_{1α} derivative for GC-MS analysis, 6-methoxymethyl-6-keto-PGF_{1α} is shown in Fig. 1 (top). A mass spectrum of the (4,4-²H₄) 6-keto-PGF_{1α} is demonstrated in Fig. 1 A. The deuterated 6-keto-PGF_{1α} contained less than 1% of the protium form. The characteristic ions of high mass in the upper mass range were m/e 602 (M-31), 562 (M-71), 512 (M-121) and 161.

The occurrence of 6-keto-PGF_{1α} in rabbit kidney medulla and cortex was demonstrated by identification of the major fragments. This is shown in Fig. 1 B by a mass spectrum of 6-keto-PGF_{1α} isolated from the renal medulla. A similar pattern was found in the renal

6-keto-PGF_{1α} standards were unstable in absolute ethanol or methanol. The order

blood flow distribution and for renin release (Larsson and Änggård 1974, Larsson *et al* 1974, Weber *et al* 1976).

In 1976 Moncada, Vane and coworkers discovered that arterial walls can synthesize a novel PG-like product from arachidonic acid. Its structure was elucidated and the compound was named prostacyclin, PGI₂ (Johnson *et al* 1976). PGI₂ is unstable in aqueous solution where it is hydrolysed to 6-keto-PGF_{1 α} . Since this is the only known pathway for 6-keto-PGF_{1 α} formation, the level of 6-keto-PGF_{1 α} in a tissue might be used to estimate PGI₂ biosynthesis (Gibson 1977).

Pace Asciak and Rangaraj (1977) demonstrated that rat renal homogenates form 6-keto-PGF_{1 α} from arachidonic acid. The physiological function of PGI₂ or 6-keto-PGF_{1 α} in the kidney is unknown. Recent studies show that exogenous PGI₂ can influence renal blood flow. PGI₂ increases renal blood flow and stimulates renin release *in vitro* and *in vivo* (Kotz *et al* 1978 a, Whorton *et al* 1977).

The present study describes the regional distribution of the endogenous formation of 6-keto-PGF_{1 α} in the rabbit kidney. Since variations in sodium intake influence renal PG synthesis in rats (Tobian and O'Donnell 1976) and urinary excretion of PGE₂ and PGF_{2 α} in rabbits (Weber *et al* 1977, Davila *et al* 1978), we also studied the urinary excretion of 6-keto-PGF_{1 α} in rabbits on low and high salt intake.

Material and methods

Materials

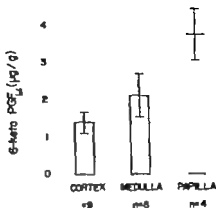
All reagents were of analytical grade. PGF_{2 α} , 6-keto-PGF_{1 α} and (3,3,4,4-³H)-6-keto-PGF_{1 α} were purchased from the Upjohn Co. (Kalamazoo, USA) through the courtesy of Dr U. Axén. The 6-keto-PGF_{1 α} standards were dissolved in ethyl acetate containing 5% methanol and stored at -20°C. The deuterated 6-keto-PGF_{1 α} contained less than 1% of the protium form according to mass fragmentography and was checked for chemical purity by thin layer chromatography. Methoxyamine HCl and BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) were obtained from Pierce, Rockford, USA. The thin layer silicic acid plates obtained from Merck (20 × 20 cm, 0.25 mm DC Fertig-Platten Kieselgel 60). Acetylsalicylic acid was obtained locally. [11-³H]-PGF_{2 α} (150 Ci/mmol) for use in a PGF_{2 α} radioimmunoassay was obtained from Radiochemical Centre, Amersham, England. The PGF_{2 α} -antibodies were raised in our laboratory and characterized as previously described (Oliw *et al* 1978). The test diets, one basal diet with 0.4% NaCl, one low salt diet with 0.03% NaCl but otherwise similar, were obtained from Ralston Purina Co., Indianapolis, USA.

Methods

Kidney analysis. Male white albino rabbits (2–2.5 kg) were kept on a standard pellet diet (Agnaf Södertälje, Sweden, containing 0.5% NaCl) and water *ad libitum*. The rabbits were killed by a blow to the head and exsanguinated. To standardize the procedure, the kidneys were removed 5 min after death and then immediately put into ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The following steps were rapidly performed at 4°C. The cortex, medulla and papilla of each kidney were macroscopically dissected and weighed (0.4–1.5 g) and chopped into small pieces. The papilla from each pair of kidneys were put in 4 volumes of the phosphate buffer and 10 µg (3,3,4,4-³H)-6-keto-PGF_{1 α} were added before homogenization in a glass tube with 5 strokes of a loose fitting Teflon pestle at 2500 r.p.m. The homogenate was centrifuged for 60 min at 90 000 g (Spinco L2 ultracentrifuge).

Proteins in the supernatant were precipitated by addition of 10 volumes of ethanol. The solvent was rapidly filtered and evaporated almost to dryness. The residue was dissolved in 2 ml water and adjusted to pH 3 with 0.1 M HCOOH and extracted with ethyl acetate (2 ml × 2). After evaporation, the extracts were purified by preparative thin layer chromatography (TLC) on silicic acid TLC plates (ethyl acetate-trimethylpentane-acetic acid-water = 110:17:20:100). Reference 6-keto-PGF_{1 α} was put in the same position on the plate. The references were separated from the sample by vertical line drawing on the

Post-mortem accumulation of 6-keto-PGF_{1α} in
of the rabbit kidney. Mean \pm S.E.



post-mortem accumulation of 6-keto-PGF_{1α} increased from cortex to medulla and
papilla (Fig. 3). The cortex contained 1.4 ± 0.3 $\mu\text{g/g}$ wet weight ($n=9$), medulla 2.1 ± 0.6
($n=8$) and the papilla 3.7 ± 0.7 $\mu\text{g/g}$ ($n=4$).

Mass analysis

occurrence of 6-keto-PGF_{1α} was demonstrated in rabbit urine by mass fragmento-
graphy. It showed the same ratio between different ion pairs, m/e 598/602 and 508/512
as retention time of the 6-keto-PGF_{1α} derivative (Fig. 2 C).

Influence of variations in dietary sodium on the urinary excretion of 6-keto-PGF_{1α}.
Urinary sodium and potassium in six rabbits is shown in Table I. On the low salt diet the
urinary excretion of Na⁺ was 0.09 ± 0.01 mmol/day. The urinary excretion of 6-keto-PGF_{1α}
was 11.8 ± 2.2 $\mu\text{g/day}$ and that of IPGF_{1α} 2.0 ± 0.6 $\mu\text{g/day}$. Treatment with acetylsalicylic
(30 mg \times 2 \times c) for two days reduced 6-keto-PGF_{1α} excretion by 71% to 3.4 ± 1.0 $\mu\text{g/day}$
(0.05) and reduced IPGF_{1α} by 85% to 0.3 ± 0.1 $\mu\text{g/day}$ ($p < 0.05$). Sodium and potassium
excretion were unchanged.

On the high NaCl intake urinary Na⁺ excretion was increased 300 fold and potassium
excretion decreased slightly (Table I). Urinary 6-keto-PGF_{1α} excretion was unchanged but
IPGF_{1α} decreased to 0.63 ± 0.10 $\mu\text{g/day}$ ($p < 0.05$).

Table I. Influence of dietary sodium on urinary excretion of 6-keto-PGF_{1α}, IPGF_{1α}, sodium and potas-
sium in rabbits ($n=6$)

	6-keto-PGF _{1α} ($\mu\text{g/day}$)	IPGF _{1α} ^b ($\mu\text{g/day}$)	Sodium ^b (mmol/day)	Potassium ^b (mmol/day)
Low NaCl intake (mean \pm S.E.)	11.8 ± 2.2	2.0 ± 0.6	0.09 ± 0.01	17.6 ± 0.6
High NaCl intake (mean \pm S.E.)	11.3 ± 4.6	0.63 ± 0.10	27.5 ± 3.4	12.8 ± 1.6
Low NaCl intake (range)	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$

^a Data of 24 h urinary excretion from 6 rabbits.

^b Data of two consecutive 24 h excretions from 6 rabbits.

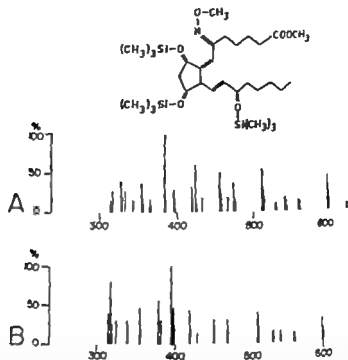


Fig. 1 Top. The structure of the 6-keto-PGF_{1α} derivative 6-methoxycarbonyl-6-methyl-1,2-bis(trimethylsilyl) ether which was used for GC/MS analysis. A. Mass spectrum of the (3,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29,30,31,32,33,34,35,36,37,38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70,71,72,73,74,75,76,77,78,79,80,81,82,83,84,85,86,87,88,89,90,91,92,93,94,95,96,97,98,99,100) 6-keto-PGF_{1α} standard B. Mass spectrum of 6-keto-PGF_{1α} from rabbit kidney medulla. Note the difference between the mass spectra of the deuterated standard (A) and the endogenous 6-keto-PGF_{1α} (B) especially in the lower mass range.

of derivatization for GC/MS analysis was therefore always methoxylation of the 4-keto group before methylation of the carboxyl-group (cf. Pace-Asciak *et al.* 1977).

Kidney analysis by mass fragmentography

The specificity of the mass fragmentographic method of 6-keto-PGF_{1α} analysis was demonstrated by identical ratios between two different ion pairs, m/e 598/602 and 508/512 at the retention time of the 6-keto-PGF_{1α} derivative. This is demonstrated by mass fragmentograms from renal cortex and renal medulla (Fig. 2 A and B).

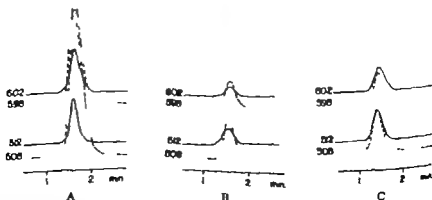


Fig. 2. Mass fragmentograms of 6-keto-PGF_{1α} using the ion pairs m/e 598/602 and 508/512 in: (A) renal medulla, (B) rabbit renal cortex, (C) rabbit urine.

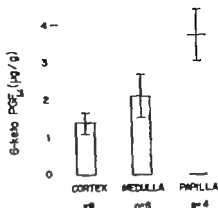


Fig. 3. Post-mortem accumulation of 6-keto-PGF_{1α} in the rabbit kidney. Mean ± S.E.

The post-mortem accumulation of 6-keto-PGF_{1α} increased from cortex to medulla and then to papilla (Fig. 3). The cortex contained 1.4 ± 0.3 μg/g wet weight ($n=9$), medulla 2.1 ± 0.6 μg/g ($n=8$) and the papilla 3.7 ± 0.7 μg/g ($n=4$).

Analysis

The occurrence of 6-keto-PGF_{1α} was demonstrated in rabbit urine by mass fragmentation which showed the same ratio between different ion pairs, m/e 598/602 and 508/512 characteristic of the 6-keto-PGF_{1α} derivative (Fig. 2 C).

The influence of variations in dietary sodium on the urinary excretion of 6-keto-PGF_{1α} and sodium and potassium in six rabbits is shown in Table I. On the low salt diet the urinary excretion of Na⁺ was 0.09 ± 0.01 mmol/day. The urinary excretion of 6-keto-PGF_{1α} was 11.8 ± 2.2 μg/day and that of IPGF_{1α} 2.0 ± 0.6 μg/day. Treatment with acetylsalicylic acid (300 mg 2 c.) for two days reduced 6-keto-PGF_{1α} excretion by 71% to 3.4 ± 1.0 μg/day ($p < 0.05$) and reduced IPGF_{1α} by 85% to 0.3 ± 0.1 μg/day ($p < 0.05$). Sodium and potassium excretion were unchanged.

On the high NaCl intake urinary Na⁺ excretion was increased 300 fold and potassium excretion decreased slightly (Table I). Urinary 6-keto-PGF_{1α} excretion was unchanged but IPGF_{1α} decreased to 0.63 ± 0.10 μg/day ($p < 0.05$).

Table I. Influence of dietary sodium on urinary excretion of 6-keto-PGF_{1α}, IPGF_{1α}, sodium and potassium in rabbits ($n=6$)

	6-keto-PGF _{1α} (μg/day)	IPGF _{1α} (μg/day)	Sodium (mmol/day)	Potassium ^b (mmol/day)
Low NaCl intake (from 2 E.)	11.8 ± 2.2	2.0 ± 0.6	0.09 ± 0.01	17.6 ± 0.6
High NaCl intake (from 2 E.)	13.3 ± 4.6	0.63 ± 0.10	27.5 ± 3.4	12.8 ± 1.6
	NS	$p < 0.05$	$p < 0.05$	$p < 0.05$

^a Mean of 24 h urinary excretion from 6 rabbits.

^b Mean of two consecutive 24 h excretions from 6 rabbits.

Discussion

The present paper confirms previous reports on the capacity of rabbit renal tissues to form 6-keto-PGF_{1α} (Pace-Asciak and Rangaraj 1977 Whorton *et al* 1978). Our main findings are post mortem accumulation of 6-keto-PGF_{1α} in the renal cortex. The level of 6-keto-PGF_{1α} is here more than 5 times higher than previously reported for PGE₂ and PGF_{2α} (Larsson and Ånggård 1976). However, in the renal medulla and papilla formation of PGE₂ dominates. Under identical experimental conditions, the renal medulla has been found to synthesize 9.2 ± 2.2 (S.D.) /g PGE₂/g (Oliw *et al* 1978) i.e. about 5 times more PGE₂ than 6-keto-PGF_{1α}. Our results therefore indicate that arachidonic acid is metabolized preferentially to primary PGs in the medulla and to 6-keto-PGF_{1α} or prostacyclin (PGI₂) in the cortex.

The level of 6-keto-PGF_{1α} in the rabbit kidney *in vivo* is likely to be much lower. Synthesis of PGs increases rapidly after death (*cf* Frölich *et al* 1976). Data indicates that the renal levels of primary PGs *in vivo* are in the low ng/g range (Larsson and Ånggård 1974).

The physiological function of PGI₂ and 6-keto-PGF_{1α} in the kidney is unknown. Renal vessels have a high capacity to synthesize PGI₂ (Moncada *et al* 1976). It is tempting to speculate that PGI₂ is formed by the renal vascular endothelium and that it could be important for the renal circulation. The effects of indomethacin on renal cortical blood flow and renal distribution were previously attributed to inhibition of the cortical synthesis of primary PGs (Larsson and Ånggård 1974 Gerber *et al* 1978 b). Since formation of 6-keto-PGF_{1α} dominates over primary PGs in the cortex, the renal circulatory effects of indomethacin could also be due to inhibition of PGI₂ synthesis. This is in agreement with the direct effect of exogenous PGI₂, which increases renal blood flow (Gerber *et al* 1978 a).

The cortical synthesis of PGI₂ might be of physiological importance for renin release. Arachidonic acid, the precursor of primary PGs and prostacyclin, increases renin release *in vivo* (Larsson *et al* 1974 Bolger *et al* 1976) and *in vitro* (Weber *et al* 1976), whereas synthesis inhibitors block renin release (Larsson *et al* 1974 Weber *et al* 1976). Interestingly, neither PGE₂ nor PGF_{2α} stimulates renin release *in vitro* (Weber *et al* 1976).

The possibility that PGI₂ is the arachidonic product which stimulates renin release recently gained experimental support *in vivo* (Gerber *et al* 1978 a) and *in vitro* (Rahmsdorf *et al* 1977). If this theory is correct one should expect the renal PGI₂ synthesis to be in parallel with the renin-angiotensin system. Sodium deprivation stimulates the renin-angiotensin system and sodium loading has the opposite effect. In the present study we found no change in urinary 6-keto-PGF_{1α} excretion when sodium excretion was increased 300 fold, while PGF_{2α} excretion decreased. This might indicate that the intrarenal PGI₂ synthesis is not influenced by variations in sodium excretion. Another possibility is, however, that urinary 6-keto-PGF_{1α} excretion to a large extent reflects the total body 6-keto-PGF_{1α} synthesis rather than the intrarenal synthesis.

The urinary excretion of PGE₂ and PGF_{2α} is generally believed to reflect the intrarenal synthesis. This assumption is based on the very low levels of these PGs in arterial blood and on the high capacity of the kidneys to metabolize PGs coming in the arterial blood and on parallel changes in renal PG synthesis and urinary PGE₂ and PGF_{2α} excretion (Frölich *et al* 1975 Dunn *et al* 1978). It is presently unknown if urinary 6-keto-PGF_{1α} reflects the intrarenal

synthesis. Recent observations indicate that PGI₁ might be a circulating hormone (Kada *et al.* 1978). Whereas PGE₂ and PGF_{2α} enter the tubular fluid in the loop of Henle (Williams *et al.* 1977), 6-keto-PGF_{1α} might also be filtered in the glomeruli and it may enter the tubular fluid in cortical parts of the nephron.

In summary the present study demonstrates a considerable relative 6-keto-PGF_{1α} synthesis in the renal cortex, while PGE₂ and PGF_{2α} are the major PG-endoperoxide products in the renal medulla. Excretion of 6-keto-PGF_{1α} in rabbit urine is 5–10 times higher than urinary PGE₂ excretion, but unlike PGF_{2α}, the 6-keto-PGF_{1α} excretion is not influenced by variations in sodium intake.

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References

1. BOYD, R. J., T. BELL, K. M. M. DONALD and R. W. SCHRELL, Evidence for an *in vivo* endogenous renal vasopressor and prostaglandin in the mammalian kidney. *J. clin. Invest.* 1975, **56**, 420–426.
2. DE, P. M., G. M. EDWARDS, P. W. RANBYLL and L. M. BLOKHOFF, Effect of prostaglandin synthesis on renal function and renal flow in the dog. *Nature (Lond.)* 1976, **259**, 244–245.
3. DE, D. T., DAVILA, E. OLIVE and E. ÅNGÖLÖD, The influence of dietary sodium on urinary prostaglandin excretion. *Acta physiol. scand.* 1979, **103**, 100–106.
4. DE, M. J., J. F. LUND and F. DIA, Renal and arteriole rates of renal excretion and excretion of prostaglandins E₂, F_{2α} and 13,14-dihydro-15-keto-P_{1α} in the dog. *Kidney Int.* 1978, **13**, 134–143.
5. DE, I. C., T. W. WALTON, B. J. SWERTMAN, M. SWOHL, A. S. NIEL, K. CARR, J. T. W. TSON and J. A. TER, Urinary prostaglandins: Identification and origin. *J. clin. Invest.* 1975, **55**, 763–770.
6. DE, I. C., B. J. SWERTMAN, K. CARR and J. A. O. TER, Prostaglandin synthesis in rabbit renal medulla. *Sci* 1976, **17**, 1103–1112.
7. DE, I. G., R. A. BLANCH, A. S. NIEL, J. D. OERLENS, H. G. BRAND, J. HOLLENFIELD and J. A. OATES, Prostaglandins and renal reflexes II: Assessment of renal secretion following infusion of PGI₂, E₂ and into the renal artery of anesthetized dogs. *Prostaglandins* 1978, **13**, 81–82.
8. DE, I. G., I. L. DE and A. S. NIEL, Enhanced renal prostaglandin production in the dog. The effect of sodium arachidonate in non-filtering kidney. *Circulat. Res.* 1978, **42**, 43–45.
9. DE, K. H., Prostaglandins, thromboxane, PGI₂, Biosynthetic products from arachidonic acid. *Chem. Rev.* 1977, **57**, 439–510.
10. DE, J. L. and J. OLLIFF, Effect of prostaglandin E₂ on the permeability response of the isolated perfused rabbit aorta to angiotensin, adenosine 3,5-bisphosphate and theophylline. *J. clin. Invest.* 1968, **47**, 154–164.
11. DE, M., Biosynthesis of prostaglandin in the renal medulla of rabbit. *FEMS Lett.* 1969, **5**, 127–130.
12. DE, R. A., D. R. MORTON, J. H. KUNIN, R. R. GORMAN, J. C. McQUINN and P. F. SUN, The chemical nature of prostaglandin X (prostaglandin). *Prostaglandins* 1976, **12**, 915–922.
13. DE, C. and E. ÅNGÖLÖD, Regional differences in the formation and metabolism of prostaglandins in the rabbit kidney. *Eur. J. Pharmacol.* 1973, **21**, 30–36.
14. DE, C. and E. ÅNGÖLÖD, Increased postarterial blood flow on stimulation of intrarenal prostaglandin biosynthesis. *Eur. J. Pharmacol.* 1974, **25**, 326–334.
15. DE, C., P. WILSON and E. ÅNGÖLÖD, Arachidonic acid secretase and indomethacin decreases plasma renin activity in the rabbit. *Eur. J. Pharmacol.* 1974, **28**, 391–394.
16. DE, C. and E. ÅNGÖLÖD, Mass spectrometric determination of prostaglandins E₂, F_{2α} and A₂ in the cortex and medulla of the rabbit kidney. *J. Pharm. Pharmacol.* 1976, **28**, 326–328.
17. DE, A. R. GUYLOR, S. BONTING and J. R. VANE, A cyclooxygenase isolated from arteries transforms arachidonic endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature (Lond.)* 1976, **261**, 663–665.
18. DE, A. R., R. KORUT, S. BONTING and J. R. VANE, Prostacyclin is a circulation hormone. *Nature (Lond.)* 1978, **273**, 767–768.

- OLIV E., I LUNDÉN and E. ÅNGOÄRD, *In vivo* inhibition of prostaglandin synthesis in rabbit liver by non-steroidal anti-inflammatory drugs. *Acta pharmacol (Kbh.)* 1978, 42, 179-184.
- PACT-ASCIAC C. R. Deuterium isotope dilution method for the specific measurement of 6-ketoprostaglandin F₁₂ by mass fragmentography. *Anal Biochem* 1977 81 251-255.
- PACT-ASCIAC C. R. Z. DOMAZET and M. CARRARA, Catabolism of 6-keto-prostaglandin F₁₂ in the kidney cortex. *Biochim. biophys. Acta (Amst.)* 1977 487 400-404.
- PACT-ASCIAC C. R. and G. RANGARAJ, Distribution of prostaglandin biosynthetic pathways in renal tissues. Formation of 6-keto-prostaglandin F₁₂. *Biochim. biophys. Acta (Amst.)* 1977 486 579-582.
- PONQ, S. S. and L. LEVINE, Biosynthesis of prostaglandins in rabbit renal cortex. *Res. Com. Clin. Pharmacol* 1976, 13 115-123.
- TORIAN L. and M. B. DONNELL, Renal prostaglandins I: relation to sodium regulation and hypertension. *Fed. Proc* 1976, 35 2388-2392.
- WEIER P. C., C. LARSSON, E. ÅNGOÄRD, M. HANBERG, E. J. CORRY, K. C. NICOLAOU and B. SCHERER, Stimulation of renin release from rabbit renal cortex by arachidonic acid and prostaglandin endoperoxides. *Circulat Res* 1976, 39 868-874.
- WEIER, P. C., C. LARSSON and B. SCHERER, Prostaglandin-C₆-9-ketoreductase as a mediator of cytokine-related prostaglandin release in interaction. *Nature (Lond.)* 1977 266, 65-66.
- WILLIAMS, W. M., J. C. FRÖLICH, A. S. NIES and J. A. OATES, Urinary prostaglandins. Site of renal tubular fluid. *Kidney Int* 1977 11 56-60.
- WHORTON, A. R., A. MINO, J. HOLLIFIELD, J. C. FRÖLICH, T. INAGAWA and J. A. OATES, Prostaglandins and renin release. I. Stimulation of renin release from rabbit renal cortical slices by PGE₂. *Prostaglandins* 1977 14 1094-1104.
- WHORTON, A. R., M. SINGEL, J. A. OATES and J. C. FRÖLICH, Regional differences in prostaglandin formation by the kidney. Prostacyclin is a major prostaglandin of renal cortex. *Biochim. biophys. Acta (Amst.)* 1978 529 176-180.

Different effects of furosemide on urinary excretion of prostaglandin E_2 and $F_{2\alpha}$ in rabbits

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Abstract

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Effect of constant infusion of furosemide (130 $\mu\text{g}/\text{min}$ i. v. for 60 min, -8) was studied on urinary excretion of water, electrolytes and immunoreactive prostaglandin E_2 (iPGE $_2$) and iPGF $_{2\alpha}$ in chloralose-anesthetized rabbits. During the furosemide infusion sodium and water excretion increased tenfold the excretion of potassium and iPGE $_2$ ca. to three times. The excretion of iPGF $_{2\alpha}$ (0.06 ± 0.03 $\mu\text{g}/100$ g kidney weight) was not significantly changed during the furosemide infusion but increased slightly after the infusion and reached a maximum (1.0 ± 0.6 $\mu\text{g}/\text{min}/100$ g) 30 to 45 min later while the increase in iPGE $_2$ excretion at this time could be attributed to cross-reaction with PGF $_{2\alpha}$. The results indicate that PGE $_2$ might possibly be involved directly in the action of furosemide, while PGF $_{2\alpha}$ might participate in sodium and water conserving mechanisms in the rabbit kidney activated by the drug induced diuresis.

Key words: Prostaglandin E_2 , prostaglandin $F_{2\alpha}$, furosemide, radioimmunoassay, sodium balance.

Furosemide belongs pharmacologically to the group of diuretics which potent natriuretic effect are mainly attributed to inhibition of an active chloride transport in the loop of Henle (Selye and Durk 1977). Furosemide also augments renal blood flow (Williamson 1975) and increases renin release *in vivo* (Vander and Carlsson 1969) and *in vitro* (Olsson and Churchill 1975).

The present paper will focus on the possible interaction between furosemide and the prostaglandin (PG) system. The renal PGs might be important in regulating renal function, sodium excretion and renin release (cf. Dunn and Hood 1977). Many observations indicate that furosemide affects the renal PGs. Williamson *et al.* (1975) demonstrated an increase in renal venous PGE following furosemide in dogs. Furosemide also increases urinary prostaglandin excretion in normal man and in hypertensive patients (Abe *et al.* 1976, Weter *et al.* 1977 a, Scherer *et al.* 1978). Evidence for a connection between the action of furosemide and the renal PGs has been obtained by the use of PG synthesis

Acetylsalicylic acid and Indomethacin were found to block the effect of furosemide on renal blood flow and renal venous PG release in dogs (Williamson *et al.* 1975, Boyel Loew 1977). Indomethacin also reduced the effect of furosemide on plasma renin activity and on sodium excretion in man (Patak *et al.* 1975, Rumpf *et al.* 1975, Frolich *et al.* 1976) and in anesthetized animals (Oliw *et al.* 1976), although the inhibition of sodium excretion is less pronounced in hydrated animals (Kövrer and Tost 1977 *cf.* Baillic *et al.* 1976).

These observations indicate that the effects of furosemide could be partly mediated by its action on renal PGs. The present report was designed to study the action of furosemide on urinary PG excretion. Rather unexpectedly we found a temporal difference in the effect of furosemide on urinary PG excretion. The increase in PGE₂ excretion was associated with the diuretic effect, whereas the increase in PGF_{2α} excretion appeared when the drug effect was over. These results indicate that PGE and PGF compounds could have different roles in the kidney.

Material and method

Materials

All reagents were of analytical grade. Furosemide (Hoechst Frankfurt, West Germany) was dissolved in 0.05 M sodium phosphate buffer pH 8.0 (1.0 mg/ml) and infused at a constant rate of 8 ml/h in 12 rabbits. Indomethacin (Mack, Sharp and Dohme, Rahway, New Jersey) was dissolved in ethanol (2.5 mg/ml). H-PGF_{2α} (150 Ci/nmol) and [³H]-PGE₂ (150 Ci/nmol) for use in radioimmunoassays were obtained from New England Nuclear, Darmstadt, West Germany. A library of antibodies against PGF_{2α} and PGE₂ conjugates were raised in rabbits and characterized as previously described (Oliw *et al.* 1978a). The test solution contained (mM): 150 NaCl, 2.3 CaCl₂ and 3.5 KCl.

Experimental

Male white New Zealand rabbits (3.0 ± 0.6 (S.D.) kg) were kept on a standard pellet diet #47 (AF Astra-Ewos, Södertälje, Sweden) and water *ad libitum*. The rabbits were anesthetized by injection into the marginal ear vein of a lukewarm Ringer solution (10 ml/kg) containing chloralose (8 mg/ml) and urethane (50 mg/ml). The rabbits were then left undisturbed for 30 min. Sometimes an additional amount (4 ml) of the anesthetic was given before a tracheal cannula was inserted. Blood pressure was monitored from the right carotid artery by means of a Statham pressure transducer (P23DC) and Grass polygraph. The left external jugular vein was used for infusion of Ringer solution at a constant rate of 0.8 ml/min throughout the experiment.

A low abdominal incision was made and the bladder was emptied. Both ureters were cannulated 1–2 cm with polyethylene tubing (Intradermic PE 50), the tip of which had been carefully blunted by scrubbing against paper. In spite of these precautions, hematuria sometimes occurred. Urine was collected in chilled glass tubes containing 50 µg indomethacin to prevent *in vitro* PG synthesis from residual elements of blood. The urine samples were centrifuged at +4°C (15 min) at the end of each collection period and frozen at the end of the experiment.

The experiment was started 60 min after beginning of the Ringer infusion. After two control periods (15 min: 2), furosemide (–8) or solvent (n=3) was infused into the marginal ear vein during 4 periods (15 min: 4) and urine was then collected for 2 periods in 3 expts. and for 6 periods in 5 expts. During this time the animals were neither artificially ventilated nor heparinized but a few ml of chloralose were sometimes needed. At the end of the experiment the kidneys were freed from surrounding tissues and weighed (mean weight 70.3 ± 4.5 (S.D.) g).

Analytical methods

Sodium and potassium in urine were measured by flame photometry using lithium as an internal standard. PGE₂ and PGF_{2α}-like immunoreactivity were measured in unextracted urine as previously described (Oliw *et al.* 1978a). PGF_{2α} was measured by radioimmunoassay (RIA) using PGF_{2α}-antiserum and the values obtained are referred to as immunoreactive PGF_{2α} (IPGF_{2α}). The assay has been validated in urine by direct comparison with GC-MS analysis with good agreement between the two methods (Oliw *et al.* 1978a).

77) For the PGE_2 analysis, the urine samples were treated with NaBH_4 converting PGE_2 to $\text{PGF}_{2\alpha}$. The latter is not naturally occurring in the rabbit and could therefore be used to estimate PGE_2 was measured by $\text{PGF}_{2\alpha}$ -antiserum and the values for immunoreactive $\text{PGF}_{2\alpha}$ are used as estimates of PGE_2 .

Sign test was used for statistical analysis (Dixon and Massey 1957) and data given as mean \pm S.E. for urinary excretion are expressed per 100 g kidney weight.

Results

Effect on sodium, potassium and water excretion

Furosemide increased urine flow and sodium excretion from 1.0 ± 0.3 and 0.16 ± 0.04 ml/min/100 g and 1.6 ± 0.2 mmol/min/100 g, respectively to a maximum of 13.0 ± 1.2 ml/min/100 g and 1.6 ± 0.2 mmol/min/100 g, respectively between 15 and 30 min of infusion (Fig. 1). Already 15 to 30 min after ending furosemide infusion, the urine flow had decreased to 2.6 ± 0.6 ml/min/100 g and sodium excretion to 0.37 ± 0.08 mmol/min/100 g.

If urine losses were not replaced but the animals were infused with 0.8 ml/min of isotonic solution. The sodium balance (sodium input minus the sodium output during the furosemide diuresis, i.e. from 30 to 105 min) was negative in 7 of 8 expts. and averaged 58 ± 0.13 mmol/min/100 g.

Sodium excretion was increased 3 times by furosemide from 0.03 ± 0.01 to a maximum of 0.15 mmol/min/100 g and decreased to control levels after the infusion (Fig. 2).

In the controls receiving solvent of furosemide, mean urinary water and sodium excretion ranged between 2.0 and 5.4 ml/min/100 g and 0.31 and 0.45 mmol/min/100 g. Mean urinary potassium excretion ranged between 0.04 and 0.07 mmol/min/100 g.

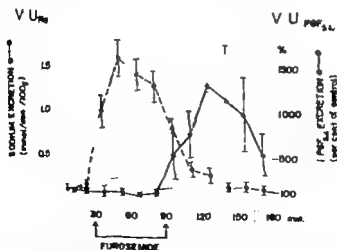


Fig. 1. The effect of furosemide infusion ($130 \mu\text{g}/\text{min}$ for 1 h) on the urinary excretion of sodium and immunoreactive PGE_2 (IPGE_2). The vertical lines mark the beginning and the end of the infusion. Sodium excretion (\bigcirc - \bigcirc). Excretion of PGE_2 (\bullet - \bullet) in per cent of the excretion in the control periods before infusion. Mean \pm S.E. (n = 5-8). The mean excretion of PGE_2 in per cent of control is indicated by the solid line.

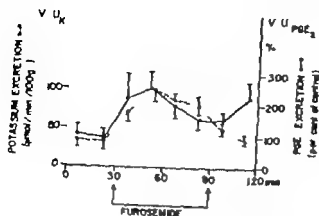


Fig. 2. The effect of furosemide infusion on the excretion of potassium and immunoreactive PGE_2 (PGE_2 is related to IPGE_2 treatment in PGE_2 and IPGE_2 is the latter measured by radioimmunoassay). Potassium excretion (O—O) and PGE_2 excretion in per cent of control (—•—). The increase in PGE_2 excretion after the furosemide infusion could be attributed to cross reaction between the PGE_2 -antiserum and IPGE_2 (cf Fig. 1 and the text).

Effect on urinary PG excretion

Urinary excretion of IPGE_2 was not significantly changed during the furosemide infusion. The $\text{IPGE}_{2\text{ex}}$ excretion was 0.06 ± 0.03 / g/min/100 g before infusion, 0.03 ± 0.01 ($p > 0.05$) after 15 to 30 min of infusion and 0.03 ± 0.01 ($p < 0.05$) at the end of the infusion. It then increased in 7 of 8 expts. and was 0.34 ± 0.19 / g/min/100 g 15 to 30 min later ($p < 0.05$) and reached a maximum of 1.0 ± 0.6 $\mu\text{g}/\text{min}/100$ g ($n = 5$) in the next urine collection period. The relative changes in IPGE_2 excretion are demonstrated in Fig. 1. There was no correlation between sodium losses during furosemide diuretics and the subsequent $\text{IPGE}_{2\text{ex}}$ excretion but IPCF excretion did not increase in the experiment with positive sodium balance during the furosemide diuretics.

Furosemide increased urinary IPGE_2 and potassium excretion in parallel (Fig. 2). The IPGE_2 excretion increased from 4.6 ± 1.3 to a maximum of 11.7 ± 2.7 ng $\text{IPGE}_{2\text{ex}}/\text{min}/100$ g after 15 to 30 min of infusion ($p < 0.05$) and was 7.1 ± 1.3 ng/min/100 g at the end of the infusion. The relative changes in IPGE_2 excretion in per cent of control is indicated in Fig. 1 (dotted line). During the marked $\text{IPGE}_{2\text{ex}}$ excretion the urinary output of IPGE_2 did not exceed 12 ng/min/100 g. However, the antiserum which was used for $\text{PGE}_{2\text{ex}}$ analysis cross-reacts to about 1% with $\text{PGF}_{2\text{ex}}$ (Oliw *et al.* 1978 a). The increase in IPGE_2 could therefore be totally attributed to cross reaction with $\text{PGF}_{2\text{ex}}$ in the samples.

In controls ($n = 3$) no significant changes were noticed in IPGE_2 and $\text{IPGE}_{2\text{ex}}$ excretion which is in agreement with previous studies (Oliw *et al.* 1978 a).

Mean blood pressure

Mean blood pressure fell slightly during the experiment from 107 ± 5 mmHg before to 93 ± 4 at the end of the furosemide infusion ($p < 0.05$) and was 92 ± 4 mmHg ($n = 3$) and 96 ± 6 mmHg ($n = 5$) 30 and 75 min later. Mean blood pressure also decreased slightly in the 3 control expts.

Discussion

PGE_2 and $\text{PGF}_{2\text{ex}}$ in urine originates from the kidneys and urinary excretion of these PGs can be used to estimate the intrarenal PG synthesis (Frolich *et al.* 1975, Williams *et al.* 1977, Dan *et al.* 1978). The present study demonstrates that furosemide increases urinary PGE_2 excretion.

2-3 times, thus considerably less than the concomitant increase in sodium and water excretion. This is in agreement with recent studies by Abe *et al.* (1977) and Scherer *et al.* (1978), who found a similar increase in PGE_2 excretion by furosemide in normal and in sodium-depleted humans. Weber *et al.* (1977a) and Scherer (1978) also found a large increase in $\text{PGF}_{2\alpha}$ excretion in normal humans. The main finding in our study is that the increase in urinary $\text{PGF}_{2\alpha}$ excretion follows *after* the furosemide diuresis. Because of long urine collection intervals in previous studies, the late increase in $\text{PGF}_{2\alpha}$ excretion might have been overlooked.

The mechanism by which furosemide increases the urinary PG excretion is likely to be complex. One explanation has been an inhibition of PG metabolism in the kidney. Thus furosemide has been proposed to reduce PG inactivation by inhibiting the enzyme 15-hydroxy-PG dehydrogenase (Paulsrud and Miller 1974; Abe *et al.* 1976). However, ID₅₀ for the inhibition of swine kidney 15-hydroxy-PG-dehydrogenase by furosemide is in the range of 10^{-4} M (Oliv *et al.* unpublished). There are also no evidence that furosemide directly affects PG metabolism *in vitro* (Gerber *et al.* 1978).

Furosemide has also been proposed to stimulate PG synthesis. Weber *et al.* (1977a) found that furosemide increased plasma arachidonate in man. They reasoned that the plasma arachidonate in turn increased the renal PG synthesis. However, this cannot explain the increase in $\text{PGF}_{2\alpha}$ in our experiments. In view of the short duration of action of furosemide the late increase in $\text{PGF}_{2\alpha}$ excretion in our study is more likely to be secondary to sodium and water losses rather than to a direct effect of furosemide. This explanation would be more consistent with the increased $\text{PGF}_{2\alpha}$ excretion in the sodium deprived animal (see below).

Previous studies have shown that dietary sodium intake influences renal PG synthesis. A low sodium chloride intake increases the intrarenal PGE_2 levels in rats and a diet high in sodium chloride decreases them (Tobian and O'Donnell 1976). Similar changes also occur in urinary PG excretion in rabbits (Weber *et al.* 1977b; Davila *et al.* 1978). Furthermore, extracellular fluid volume expansion with Ringer solution decreases urinary $\text{PGF}_{2\alpha}$ excretion in rabbits (Oliv *et al.* 1978b). These observations indicate that the late increase in $\text{PGF}_{2\alpha}$ excretion following furosemide might be related to the negative sodium balance. The antinatriuretic action of the renal PGs has recently been proposed (Tobian and O'Donnell 1976; Kirschenbaum and Stein 1976, 1977).

Another possibility is that furosemide could also increase PG synthesis by stimulating the renin-angiotensin system. Angiotensin II has been found to increase urinary PG excretion *in vivo* (Frölisch *et al.* 1975; Dunn *et al.* 1978) and PG synthesis *in vitro* (Danon *et al.* 1975). The renin-angiotensin system, as well as anacethesis and other vasoconstrictor stimuli, might therefore contribute to the increased urinary PG excretion in our study (Scherer *et al.* 1978).

In the present study furosemide increased potassium and PGE_2 excretion in parallel. However, this might not be a causal relationship, since urinary PGE_2 and $\text{PGF}_{2\alpha}$ excretion is not changed by potassium deprivation in rats (Hood and Dunn 1978).

In summary, our data suggest that the early increase in PGE_2 excretion could be related to the effects of furosemide on renal blood flow (Williamson *et al.* 1975; Berg and Loew

1977) The late and large increase in urinary PGF_{α_2} excretion could be attributed to renal compensation of sodium and water losses. The major mechanism of furosemide is to shift the electrolyte concentration gradient in the medulla by blocking an active chloride transporter (Olsen 1975). Indomethacin on the other hand increases this gradient (Ganguli *et al.* 1975). It is therefore tempting to speculate that PGF_{α_2} is somehow related to the maintenance of the electrolyte gradient in the renal medulla.

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References

- ABE, K., Y. OTSUBA, M. YASUHIMA, S. CHIRA, M. SENO, N. IZOKAWA and K. YOSHINAGA, Metabolic conversion of PG in man. Effect of furosemide on the excretion of the main metabolite of PGF_{α_2} . *Prostaglandins* 1977, 14, 843-848.
- ABE, K., M. Y. TSUHIMA, S. CHIRA, N. IZOKAWA, T. ITO and K. YOSHINAGA, Effect of furosemide on urinary excretion of prostaglandin E_2 in normal volunteers and patients with essential hypertension. *Prostaglandins* 1977, 14, 317-321.
- BALLIE, M. D., K. CROSSMAN and J. B. HOOK, Natriuretic effect of furosemide after inhibition of prostaglandin synthetase. *J. Pharmacol. exp. Ther.* 1976, 199, 469-476.
- BERG, K. J. and B. LOEW, Inhibition of furosemide-induced natriuretic by acetylsalicylic acid in rats. *Scand. J. Clin. Lab. Invest.* 1977, 37, 1-5-131.
- DANON, A., L. C. T. CHANG, B. J. SWETTMAN, A. S. NIEZ and J. A. OATES, Synthesis of prostaglandin in the rat renal papilla *in vivo*. Mechanism of stimulation by angiotensin II. *Biochem. biophys. Acta* (1975) 333, 71-81.
- EL VILA, D., T. DAVILA, E. OLJW and E. ÅNGÅRD, The influence of dietary sodium on urinary prostaglandin excretion. *Acta ph. med. scand.* 1978, 103, 100-106.
- DIXON, W. J. and F. J. MASSEY, *Introduction to statistical analysis*. McGraw-Hill Book Company Inc. 1957.
- DUNN, M. J. and V. L. HOOD, Prostaglandins and the kidney. *Amer. J. Physiol.* 1977, 233, 169-174.
- DUNN, M. J., J. F. LIARD and F. DRAY, Basal and stimulated rates of renal secretion and reabsorption of prostaglandins E_2 , F_2 and 13,14-dihydro-15-keto- F_2 in the dog. *Kidney Int.* 1978, 13, 136-147.
- FRÖLICH, J. C., T. W. WILSON, B. J. SWETTMAN, M. SUGIEL, A. S. NIEZ, K. CARR, J. T. WATSON and J. A. OATES, Urinary prostaglandins. Identification and origin. *J. Clin. Invest.* 1975, 55, 763-770.
- FRÖLICH, J. C., J. W. HOLLIFIELD, J. C. DORMORE, B. L. FRÖLICH, H. SEYBERTH, A. M. NEWLAND and J. A. OATES, Suppression of plasma renin activity by indomethacin in man. *Circulat. Res.* 1975, 37, 447-455.
- GANGULI, M., L. TOBIAN, S. AZAR and M. O'DONNELL, Evidence that prostaglandin synthesis increases the concentration of sodium and chloride in rat renal medulla. *Circulat. Res.* 1977, 40, Suppl. 1, 135-139.
- GERBER, J. G., W. C. HUBBARD, R. A. BRANCH and A. S. NIEZ, The lack of an effect of furosemide on uterine prostaglandin metabolism *in vivo*. *Prostaglandins* 1978, 15, 663-670.
- HOOD, V. L. and M. J. DUNN, Urinary excretion of prostaglandin E_2 and prostaglandin $\text{F}_{2\alpha}$ in potassium deficient rats. *Prostaglandins* 1978, 15, 73-80.
- KIRSCHENBAUM, M. A. and J. H. STEIN, The effect of inhibition of prostaglandin synthesis on urinary sodium excretion in the conscious dog. *J. Clin. Invest.* 1976, 57, 517-521.
- KIRSCHENBAUM, M. A. and J. H. STEIN, The effect of prostaglandin inhibition on sodium excretion during expansion of the extracellular fluid of man. *J. Lab. Clin. Med.* 1977, 90, 46-56.
- KÖVLA, G. and H. TOST, The effect of indomethacin on kidney function. Indomethacin and haemodynamic antagonism. *Pflügers Arch. ges. Physiol.* 1977, 372, 215-220.
- LYONS, H. J. and P. C. CHU CHILL, Renal secretion from rat renal cortical cell suspensions. *Am. J. Physiol.* 1975, 228, 1835-1839.
- OLJW, E., G. KÖVLA, C. LARSSON and E. ÅNGÅRD, Reduction by indomethacin of furosemide effect on the rabbit. *Europ. J. Pharmacol.* 1976, 38, 95-100.
- OLJW, E., I. LUNDÉN and E. ÅNGÅRD, *In vivo* inhibition of prostaglandin synthesis in rabbit kidney by non-steroidal anti-inflammatory drugs. *Acta pharmacol. (Kbh.)* 1978, 42, 179-184.

27. E. H. KAYE, C. LARSON and E. ÅNGSTRÖM, Indomethacin and ibuprofen sodium increase sodium/water excretion after extracellular volume expansion in the rabbit. *Europ. J. Pharmacol.* 1978 b. 49-58.
28. U. B. Observations of the early diuretic response after intravenous administration of bumetanide and furosemide in dogs. *Acta physiol. scand.* 1973. 93. 195-201.
29. R. Y. B. K. MOOREHEAD, C. J. BEVILL, P. E. HYBERT M. BAKER and J. B. LEE, Antagonism of the effect of furosemide by indomethacin in normal and hypertensive man. *Prostaglandins* 1975. 10. 649-659.
30. J. R. and G. N. MILLER, Inhibition of 15-OH prostaglandin dehydrogenase by several diuretic agents. *Fed. Proc.* 1974. 33. 590.
31. K. W. S. FERNIE, H. D. LOWIE and F. SCHLES, The effect of indomethacin on plasma renin activity in man under normal conditions and after stimulation of the renin-angiotensin system. *Prostaglandins* 1975. 10. 641-647.
32. B. J. SCHNEIDERMAN, M. BOROMIS and P. C. WEISS, Prostaglandin (PG) analysis in urine of man and rat by different radioimmunoassays: Effect on PG-excretion by PG synthetase inhibitors, aspirin and furosemide. *Prostaglandins* 1978. 15. 255-266.
33. J. P. and J. H. DOLZ, Site of action of diuretic drugs. *Kidney int.* 1977. 11. 1-8.
34. L. and M. O'DONNELL, Renal prostaglandins in relation to sodium regulation and hypertension. *J. Proc.* 1976. 35. 2383-2392.
35. A. J. and J. CARLSON, Mechanism of the effects of furosemide on renal secretion in anesthetized guinea pigs. *Circulat. Res.* 1969. 25. 145-152.
36. P. C., B. SCHNEIDER and C. LARSON, Increase of free arachidonic acid by furosemide in man as cause of prostaglandin and renal release. *Europ. J. Pharmacol.* 1977. 41. 329-332.
37. P. C., C. LARSON and B. SCHNEIDER, Prostaglandin E_2 -9-ketoreductase as mediator of salt intake and prostaglandin-renal vasoconstriction. *Nature (Lond.)* 1977 b. 266. 65-66.
38. W. M. J. C. FEELICH, A. S. NIX and J. A. OATES, Urinary prostaglandins. Site of entry into renal tubular fluid. *Kidney int.* 1977. 11. 256-260.
39. J. JARON, H. E. W. A. BOOTLAND, G. R. MARCHANT, H. B. FARLEY and D. E. ORDEN, Furosemide local release of prostaglandin E to increase renal blood flow. *Proc. Soc. exp. Biol. (N.Y.)* 1975. 150. 4-106.

Fasting and prolonged exercise increase vasoactive intestinal polypeptide (VIP) in plasma

By

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Abstract

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Fasting and prolonged exercise increase vasoactive intestinal polypeptide (VIP) in plasma Acta physiol. scand. 1979 105 374-377

6 young men had venous blood drawn during 4 experiments. The concentration of VIP in plasma increased markedly (from 1.8 (0-4.5) to 3.3 (7.8-43.1) pmol l⁻¹ mean and range) during 3 h of mild to moderate exercise but not at all during an equivalent period of rest or during short term maximal exercise. During 59 h of fasting, VIP increased from 3.6 (0.6-6.6) to 10.2 (6.6-18.1) pmol l⁻¹ (p < 0.05). The concentration of glucose in plasma decreased significantly during the prolonged exercise as well as during fasting. The known metabolic actions of VIP and the demonstrated increases in its plasma concentration during negative energy balance indicate that VIP is a polypeptide of substrate origin.

Vasoactive intestinal polypeptide (VIP) was originally isolated from porcine small intestine (Said and Mutt 1970). It is considered as a possible gastrointestinal hormone (Rivier, Müller and Thompson 1976 a, b, Johnson 1977) and since recently as a possible neurotransmitter (Bryant *et al* 1976, Larsson *et al* 1976). Because of similarities in amino acid sequence and pharmacology VIP has been grouped together with glucagon, secretin and gastric inhibitory polypeptide into the secretin-glucagon family (Rayford, Miller and Thompson 1976 a, b, Johnson 1977). However the physiological role of VIP is not established (Rayford, Miller and Thompson 1976 a, b, Bryant *et al* 1976, Johnson 1977). In order to clarify the physiological role of a hormone it is necessary to elucidate the conditions for its release. The concentration of glucagon in plasma has been shown to increase during muscular exercise (Galbo, Holst and Christensen 1975) and fasting (Markey *et al* 1970). In order to extend the knowledge about the physiology of VIP we have now measured its concentration in plasma during these strains.

Materials and methods

6 healthy males (25 (17-31) yrs (mean and range), 183 (176-187) cm and 77 (61-81) kg), familiar with the laboratory and the applied procedures, volunteered in the study. At intervals of 1-7 weeks they participated in 4 different experiments the sequence of which was varied. One morning the subjects exercised 3 h at

ally tested bicycle ergometer at a load (70-147 W) calculated to require 40% of the individual maximal oxygen uptake ($\dot{V}O_2$ max, 56 (52-59) and $\text{kg}^{-1} \text{min}^{-1}$) determined previously on treadmill. During the exercise the subjects rested in a chair for an identical period of time. Still another morning the subjects performed two successive treadmill runs (inclination 7%) lasting 10 and 5 min and previously not to require 60 (speed 6.4-7.5 km h^{-1}) and 100 m (9.5-11.7 km h^{-1}), respectively of individual maximal velocity. Finally the subjects were examined after 39 h fast during which they were not allowed to smoke, exercise in sports and only consumed water, mineral water, vitamins and tea. The subjects always slept in the laboratory after a good night's sleep, at least 10 h fast (only taking in water) and tobacco and after at least 31 h alcohol and sports abstinence. They are weighed and had catheter inserted in the brachial vein, however they rested 30 min before the first blood sample was drawn. (1) During exercise the catheter was advanced to the subclavian vein. The electrocardiogram was recorded with precordial electrodes. Blood for VIP determinations was collected in ice tubes containing EDTA and 500 IU of aprotinin per ml of blood. After centrifugation at 4°C plasma was stored at -20°C until analysed by specific, sensitive and precise radioimmunoassay (Fahrenkrug and Schaffhitzky 1977). Glucose (Schmidt 1961) and glycerol (Eggstein 1966) were determined by enzymatic spectrometric methods and FFA spectrophotometrically as copper soaps (Dincoffe 1964). Hematocrit values were determined by the microhematocrit method. Statistical evaluation of the data was made by means of Wilcoxon nonparametric ranking test for paired data and Spearman rank correlation analysis (Siegel 1956).

Results

During the prolonged exercise the concentration of VIP in peripheral plasma increased markedly (Fig. 1). Simultaneously plasma glucose decreased (Fig. 1) and was at the end of the exercise inversely correlated with the markedly increased VIP concentrations ($r_s = -0.846$, $p < 0.05$). Heart rate increased throughout the exercise and was 135 (133-138) and 145 (142-148) beats min^{-1} ($p < 0.05$) in the 30th and in the 180th min, respectively. The hematocrit increased slightly from 43.9 (40.7-47.3)% at rest to 45.5 (42.5-48.1)% at the end of the exercise ($p < 0.05$). During the 3 h of rest in the control expt. the measured parameters did not change significantly.

During short term moderate and heavy exercise neither VIP nor glucose concentrations were changed significantly. VIP concentrations were 3.6 ± 0.9 , 3.7 ± 1.1 and 4.4 ± 1.1 nmol l^{-1} mean and S.E. at rest, submaximal and maximal exercise, respectively while glucose concentrations were 5.2 ± 0.1 , 5.1 ± 0.2 and 5.2 ± 0.2 mmol l^{-1} . Heart rate increased to 176 ± 4 at rest to 152 ± 6 and 189 ± 2 beats min^{-1} during submaximal and maximal exercise, respectively while the hematocrit was 43.4 ± 0.8 , 45.3 ± 0.8 and 47.0 ± 0.8 %, respectively ($p < 0.05$).

After 39 h of fasting the subjects had lost 3 (-4) kg of body weight and had pronounced ketonuria measured by nitro (Ames). The concentrations in serum of FFA (from 0.302 (0.0-0.491) to 0.980 (0.622-1.742) meq l^{-1}) and glycerol (from 57 (39-67) to 178 (122-281) mg l^{-1}) had increased ($p < 0.05$). Also VIP concentrations in plasma had increased markedly while glucose concentrations had decreased (Fig. 2). Hematocrit was 43.4 ± 0.8 % at rest and 45.7 ± 1.3 % after the fast ($p < 0.01$).

Discussion

The present study has shown that the concentration of immunoreactive VIP in peripheral plasma increases during fasting and during prolonged exercise of an intensity equivalent to everyday activities. During short term moderate or heavy exercise, however VIP

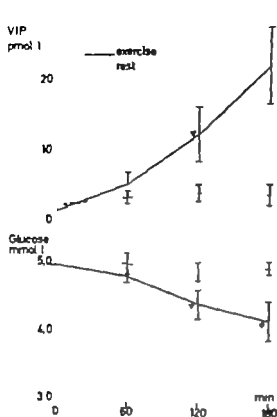


Fig. 1

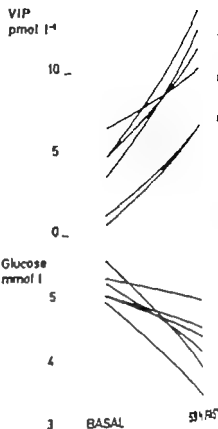


Fig. 2

Fig. 1 Mean plasma concentrations (\pm S.E.) of vasoactive intestinal polypeptide (VIP) and of glucose in 6 subjects during rest and prolonged exercise are plotted against time. ∇ denotes value significantly different from initial value ($p < 0.05$). ∇ denotes value significantly different from value obtained after an equal period of rest ($p < 0.05$).

Fig. 2. The plasma concentrations of vasoactive intestinal polypeptide (VIP) and of glucose in standing subjects after a: overnight fast (Basal) and after 59 h of fasting.

concentrations did not change significantly. Similar findings have been made in the case of glucagon (Marlias *et al.* 1970; Galbo, Holst and Christensen 1975). Since VIP is widely distributed in the body (Bryant *et al.* 1976; Larsson *et al.* 1976) the origin of the VIP released causing the increased concentrations in plasma can not be pointed out with certainty. However, the highest tissue concentrations of VIP have been found in the gastrointestinal tract (Bryant *et al.* 1976; Larsson *et al.* 1976). VIP may be released from VIP-containing nerve fibers (Bryant *et al.* 1976; Larsson *et al.* 1976) and endocrine cells (Bryant *et al.* 1976). The fact that VIP-immunoreactive endocrine cells have not been detected in the pancreas by the VIP antiserum used to measure VIP concentrations in the present study (Larsson *et al.* 1976) indicates that VIP in plasma derived from nerve fibers.

The consequences of an increased release of VIP during exercise and fasting can not be pointed out with certainty either. The pharmacology of VIP comprises many actions (Rayford, Miller and Thompson 1976a, b; Johnson 1977). Among these stimulating glucagon secretion (Ohneda *et al.* 1977), lipolysis (Frandsen and Moody 1973), and hypotension.

glycolysis and gluconeogenesis (Matsumura, Akiyoshi and Fujii 1977) appear most apt in relation to the observed increases in VIP concentrations during exercise and rest. Certainly VIP is hardly produced in the liver (Bryant *et al.* 1976) but since VIP is rapidly inactivated on hepatic transit (Rayford, Müller and Thompson 1976a), the destruction of VIP in portal blood draining the gastrointestinal tract possibly in the present study was higher than in peripheral blood and sufficient to stimulate hepatic glycolysis and gluconeogenesis. Higher concentrations of VIP in portal than in peripheral blood have previously been found in anesthetized pigs (Fahrenkrug *et al.* 1978). The known biological actions and the finding in the present study of increased plasma concentrations during negative energy balance indicate that VIP like glucagon, has a biological role as "a polypeptide of substrate need".

References

- BLUM, M. G. & R. BLOOM, J. M. POLAK, R. H. ALBUQUERQUE, I. MODLIN and A. G. E. PEARSE, 1976, A possible dual role for vasoactive intestinal peptide as gastrointestinal hormone and neurotransmitter. *Lancet* 1976, 2, 991-993.
- COHEN, W. G. The colonic micro-determination of non-esterified fatty acids in plasma. *Chin. Chim. Acta* 1964, 9, 122-134.
- FRUM, M. Eine neue Bestimmung der Neutralfette im Blutserum und Gewebe. *Klin. Wochenschr.* 44, 4, 267-272.
- FRUM, J. and O. B. SCHAFFALITZKY DE MUCKADELL, Radioimmunoassay of vasoactive intestinal polypeptide (VIP) in plasma. *J. Lab. clin. Med.* 1977, 89, 1379-1388.
- FRUM, J., H. GALLI, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL, Influence of the autonomic nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *J. Physiol. (Lond.)* 1978, 280, 405-422.
- GAO, E. K. and A. J. MODY, Lipolytic action of newly isolated vasoactive intestinal polypeptide. *Am. J. Med. Res.* 1973, 5, 196-199.
- GAO, E. K., J. J. HOLST and M. J. CHRISTENSEN, Glucagon and plasma catecholamine responses to graded and prolonged exercise in man. *J. appl. Physiol.* 1975, 38, 70-76.
- GAO, E. K. Gastrointestinal hormones and their functions. *Ann. Rev. Physiol.* 1977, 39, 135-152.
- GAO, E. K., J. FAHRENKRUG, O. SCHAFFALITZKY DE MUCKADELL, P. SCHÖLLER, R. HÄGEMAN and J. F. RUTILE, Localization of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proc. nat. Acad. Sci. USA* 1976, 73, 3197-3200.
- GAO, E. K., Y. T. AOKI, R. H. UNGER, J. S. SOLENER and G. P. CAHILL, JR. Glucagon levels and metabolic effects in fasting man. *J. clin. Invest.* 1970, 49, 236-270.
- GAO, E. K., H. ASTRUP and S. PETER, Effects of gastrointestinal and related hormones on hepatocytes and gluconeogenesis in cultured liver cells. *J. Biochem.* 1977, 82, 1073-1076.
- GAO, E. K., S. ICHIKAWA, K. HORIKAWA, M. CHIBA, T. SAKAI, Y. KAI, K. W. CHAN and S. YAMAGA, Effect of intrapancreatic administration of vasoactive intestinal peptide upon the release of insulin and glucagon in dogs. *Horm. Metab. Res.* 1977, 9, 447-452.
- GAO, E. K., T. A. MILLER and J. C. THOMPSON, Secretin, cholecystokinin and newer gastrointestinal hormones. *A. Engl. J. Med.* 1976, 294, 1093-1101.
- GAO, E. K., T. A. MILLER and J. C. THOMPSON, Secretin, cholecystokinin and newer gastrointestinal hormones. *A. Engl. J. Med.* 1976, 294, 1157-1164.
- GAO, E. K. and V. MUTT, Polypeptide with broad biological activity: Isolation from small intestine. *Proc. nat. Acad. Sci. USA* 1970, 66, 1217-1218.
- GAO, E. K. and F. H. DE VRIES, Die enzymatische Bestimmung von Glucose und Fructose nebeneinander. *Klin. Wochenschr.* 1961, 39, 1, 44-47.
- GAO, E. K., *Nonparametric statistics for the behavioral sciences*. Tokyo, McGraw-Hill Kogakusha, Ltd. 1964, pp. 312.

Effect of terbutaline on lecithin content in alveolar lung wash in fetal rabbits

By

B BERGMAN, T HEDNER and P LUNDQVIST

Adrenergic beta-receptor stimulating drugs have been used extensively during the last years in the treatment of threatening preterm labour. There has also been a considerable interest in the effects of these drugs on the fetus and neonate, especially on the lung function of the premature infant. Clinical studies indicate a reduced incidence of respiratory distress syndrome in premature infants born of mothers who have been treated with beta-agonists, such as isoxuprine or ritodrine (Kero *et al* 1973; Boog *et al* 1975). Bergman and Hedner (1978) used a more selective beta₂-receptor stimulating drug, terbutaline, in an attempt to arrest preterm labour and found a reduced incidence of hyaline membrane disease in a series of preterm infants.

Wyszogrodski and associates (1974) demonstrated improved pulmonary stability by pressure-volume experiments on lungs of isoxuprine injected fetal rabbits. We have found an increased pulmonary distensibility in similar experiments after terbutaline administration into fetal rabbits 3 h before preterm delivery (Bergman *et al* 1978). These results indicate an increased pulmonary maturation after administration of beta mimetics. However, a more direct way to assess the effect of these drugs on pulmonary maturation is to measure certain biochemical parameters of surfactant in the fetal lung. Enhörning *et al* (1977) investigated the surfactant properties after isoxuprine administration to fetal rabbits and found an increased lecithin/sphingomyelin ratio of the fetal pulmonary fluid.

The purpose of the present study was to investigate the effect of terbutaline on the lecithin content in alveolar wash from the preterm rabbit lung.

New Zealand white rabbits, weighing between 4.1 and 4.5 kg, were mated under direct observation 28 days before gestation. Half of the does received an intramuscular injection of 2.5 mg terbutaline per kg. The control animals received an equal volume of vehicle. 3 h after the injection the does were anaesthetized with ether and a laparotomy was performed. The fetuses were removed, immersed in normal saline and allowed to breathe during this procedure. After tracheotomy a cannula was inserted and the lungs were expanded with 2 ml air. This was followed by 6 gentle lavagings, each with 1 ml of 0.9% saline (Cl). The cumulative lecithin recovery by this method is approximately 95% (Gluck *et al* 1967). Lung lecithin was extracted with chloroform, methanol (1:2). The chloroform layer was chromatographed on thin-layer silica gel plates. The lecithin spots were charred by spraying with copper acetate reagent and concentration of lecithin determined by densitometric scanning (Zelcer *et al* 1969).

1. Details of the material.

	Control	Terbutaline
no. of does	3	3
no. of fetuses	16	23
weight (mean \pm S.E.)	42.2 \pm 0.8	43.2 \pm 1.3

No significant difference in fetal weight was found between the two groups. The mean weight was 43.2 g in fetuses of terbutaline treated mothers compared to 42.2 g in the controls (Table I).

The total lecithin of pulmonary lavage of fetuses born of terbutaline-treated does was distinctly higher ($p < 0.001$) than the total lecithin in the lavage fluid from fetuses of saline treated does (Fig. 1).

The lung ash lecithin content is known to reflect the pulmonary biochemical maturation in the fetal rabbit. The lecithin level in pulmonary washings increased by approximately 50% after terbutaline administration to the does, which is in agreement with the corresponding results of Enhorning *et al.* (1977) who administered isoprenaline directly into the lungs. It has also been shown that beta-receptor stimulating drugs, such as terbutaline, when given to the pregnant rabbit, reaches the fetuses in small amounts (Bergman *et al.* unpublished observations).

The exact mechanism whereby terbutaline affects the maturation of the fetal lung is not known at present. The data achieved may be the result of a variety of mechanisms. Beta-adrenergic drugs are well known to effect a dilatation of the lower airways and recently also a dilatation of the fetal lungs has been demonstrated (Enhorning *et al.* 1977). These two factors may facilitate neonatal respiratory adaptation. However the increased amount of total lecithin recovered from pulmonary lavage could hardly be explained by dilatation and hydration. It seems more logical to assume that the increased lecithin levels reflect the mechanism earlier proposed, namely that of a release of intracellularly stored surfactant into the alveolar space. This explanation seems attractive in view of the fact that the fetal lung at this stage of gestation is morphologically mature, but that surfactant is still mainly

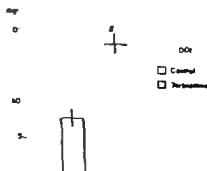


Fig. 1. Effect of maternal terbutaline on total lecithin in alveolar lung wash of fetal rabbits. Shown are mean \pm S.E. Figures indicate the number of experiments.

stored intracellularly (Wyszogrodski *et al.* 1974, Kikkawa *et al.* 1968). The phenomena described can also be explained by an increased biosynthesis of pulmonary lecithin as by-receptor stimulating agents are known to increase tissue cyclic adenosine monophosphate, which could lead to enhanced pulmonary lecithin synthesis (Barrett *et al.* 1976).

If the physiological situation and the effect of terbutaline are similar in the human neonate there seems to be reason to consider this beneficial influence of beta-mimetics by the treatment of preterm labour and also when evaluating the effect of corticoid treatment on lung maturation. The possible clinical value of terbutaline in this respect is supported by the results of a recent clinical report (Bergman and Hedner 1978). However before definite conclusions are made it should also be born in mind that any beneficial effects of beta-mimetics depend on the placental passage as related to dose.

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References

- BARRETT, C. T., A. SEVANTIAN, N. LAVIN and S. A. KAPLAN, Role of adenosine 3',5' monophosphate in maturation of fetal lungs. *Pediatr Res* 1976, 10, 621-625.
- BERGMAN, B. and T. HEDNER, Antepartum administration of terbutaline and the incidence of fetal membrane disease in preterm infants. *Acta Obstet Gynec Scand* 1978, 57, 217-221.
- BERGMAN, B., T. HEDNER and P. LUNDBORG, Effect of terbutaline on the pressure-volume relationship in fetal rabbit lung. *Acta Obstet Gynec Scand* 1978, 57, 323-326.
- BOOD, G., M. BEN BRAHIM and R. QANDAR, Beta-adrenergic drugs and possible prevention of respiratory distress syndrome. *Brit J Obstet Gynaecol* 1975, 82, 286-288.
- ENIÖRNING, G., D. CHAMBERLAIN, G. CONTRERAS, R. BURGOYNE and B. ROBERTSSON, Isoxuprine-induced release of pulmonary surfactant in the rabbit fetus. *Amer J Obstet Gynec* 1977, 129, 197-200.
- FEWSTER, M. L., B. J. BURNS and J. F. MEAD, Quantitative densitometric (thin-layer) chromatography of lipids using copper acetate reagent. *J Chromatogr* 1969, 43, 120-126.
- GLUCK, L., M. SRISENEY and M. V. KULOVICH, The biochemical development of surface activity in mammalian lung. II. The biosynthesis of phospholipids in the lung of the developing rabbit fetus and newborn. *Pediatr Res* 1967, 1, 247-265.
- KEO, P., T. HIRVONEN and I. VÄLIMÄKI, Prenatal and postnatal isoxuprine and respiratory distress syndrome. *Lancet* 1973, 2, 198.
- KIKKAWA, Y., E. K. MOTOYAMA and L. GLUCK, Study of the lungs of fetal and newborn rabbits. Morphologic, biochemical and surface physical development. *Amer J Pathol* 1968, 52, 177-209.
- WYSZOGRODSKI, I., H. W. TARUCHI and M. E. AVERY, Isoxuprine-induced alterations of pulmonary pressure-volume relationships in premature rabbits. *Amer J Obstet Gynec* 1974, 119, 1107-1110.

Effects of angiotensin II on cerebral blood vessels

By

L. EDVINSSON, J.-E. HARDERÖ and CH. OWMAN

Octapeptide angiotensin II exerts a potent constrictor action on vascular and non-vascular smooth muscle (Page and Brumpton 1974, Page and McCubben 1968) by a direct action on smooth muscle and by a potentiating effect of adrenergic nerve activity (Zimmermann 1978). Angiotensin receptors have been demonstrated on the plasma membrane of many target cells (Altura and Altura 1977, Baudouin *et al.* 1972, Devynck *et al.* 1973, LeMoult and Paley 1975). Besides the direct vasoconstrictor action of angiotensin other central actions of the peptide following intraventricular infusions have been demonstrated (see Edvinsson *et al.* 1977): an antidiuretic hormone release, drinking and a centrally mediated nociceptive response. This is in accordance with recent demonstrations of the existence of angiotensin II-containing nerve terminals in brain (Fuxe *et al.* 1976). This would indicate a direct action of angiotensin II on brain vessels whereby central release of the peptide could induce a local vasoconstriction. The peptide has for a long time been used in studies of the cerebral blood flow, particularly in the phenomenon of autoregulation, which is the basic ability of the brain to maintain a constant flow despite relatively marked changes in perfusion pressure (see Edvinsson and MacKenzie 1976). In such studies it is assumed that angiotensin II, used to induce the desired degree of hypertension, is in itself inert with respect to the cerebral circulation. Evidence that this is not true has been obtained in the present study of a model utilizing isolated middle cerebral arteries from cats on which the vasoconstrictor action induced by angiotensin II has been tested and the response compared with that of other vasoactive agents.

Isolated segments (5 mm long, 300-400 μ m in diameter) of cat's middle cerebral artery were obtained from 6 adult cats of either sex (3 cats were subjected to cranial sympathectomy 2 weeks beforehand). The animals were exsanguinated under nembutal anaesthesia (30 mg/kg i.p.), the brain was removed, and the vessels immediately dissected out and placed in aerated Krebs-Ringer buffer solution of the following composition (mM concentrations): NaCl 118, KCl 4.5, MgSO₄ 2H₂O 1.0, KH₂PO₄ 1.0, NaHCO₃ 25, CaCl₂ 2.0, H₂O 1000, glucose 10. Two preparations were mounted between metal prongs in the same mounted organ bath, continuously aerated with 95% O₂ and 5% CO₂ to give a pH between 7.3 and 7.4. Circular mechanical activity was measured by Endevco Model 8107-2 force-displacement transducers and recorded on a Grass Model 7B polygraph. The technique has previously been described in detail (Edvinsson *et al.* 1974, Edvinsson and Owman 1974).

Administration of angiotensin II amide (Hypertensin, CIBA) into the organ bath induced

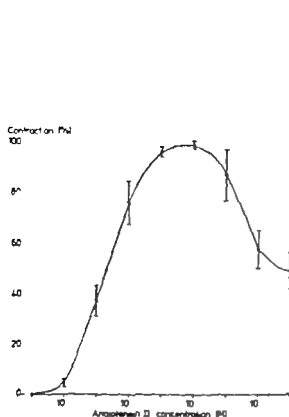


Fig. 1

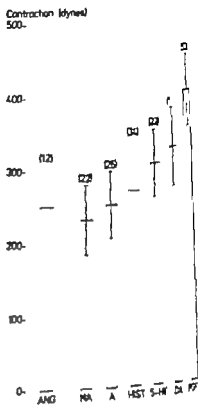


Fig. 2

Fig. 1 Log dose-response relationship following cumulative applications of angiotensin II to cerebral arteries in 12 expts. The values show mean contractions (expressed as percent of the maximum response) \pm S.E.

Fig. 2. Comparison between maximum contractile effects of angiotensin II (ANG) and (list of substances): (NA) adrenaline (A), histamine (HIST), 5-hydroxytryptamine (5-HT), dopamine (DA) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) compiled from data obtained in previous studies (Edvinsson and Owman 1977). Values are means \pm S.E., number of expts. within parenthesis.

a prompt constriction of the isolated segments of the middle cerebral artery. The response was the same irrespective of whether the vessels had been sympathectomized or not both hands. Therefore all dose-response curves were grouped together. A constriction was observed already at a concentration of 10^{-10} M of angiotensin and the maximum effect was obtained with 10^{-7} M. In concentrations above 10^{-7} M the contractile effect was less marked (Fig. 1). The mean concentration of angiotensin at which half maximum response was obtained ($5.66 \pm 1.44 \cdot 10^{-9}$ M (mean values \pm S.E.)) and the maximum contractile effect was 261 ± 61 dynes. Tachyphylaxis often occurred when repeated dose-response curves were obtained at short time intervals. The contraction did probably not involve an interaction with presynaptic α receptors since in 2 vessels the angiotensin-induced contraction was the same as controls after pretreatment during 30 min with 10^{-6} M phenoxybenzamine.

Angiotensin II is of interest not only by itself but also in comparison with other vasoconstrictor hormones. This peptide was slightly less effective as a contractile agent (see Fig. 2) than 5-hydroxytryptamine, dopamine and prostaglandin $F_{2\alpha}$. Angiotensin II is commonly

— levating the systemic blood pressure when studying not only the pressure flow relation in the cerebral circulation, but also the impaired structural blood-brain barrier function that may follow an acute rise in systemic pressure. In such experiments it is assumed angiotensin has little or no action on the cerebral arteries. However a pronounced action was obtained with angiotensin II in isolated brain vessels, the effect being accompanied by a gradual acceleration of spike activity (Lusamvuku *et al* 1979). Recently occlusion was demonstrated to be a potent constrictor of pial precapillary vessels (Wei 1971). These findings suggest a role of angiotensin II on cerebral vessels *in situ* and a likely modulatory involvement in studies on cerebral blood flow autoregulation, although permeability across the blood-brain barrier is poor (Vollicer and Loew 1971)

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References

- ALL, R. M. and B. T. AUSTIN, Vascular smooth muscle and neurophysical hormones. *Federation Proc* 1973 32 1853-1860.
- BORTH, M. P. MEYER, S. FERNANDEZ and J. L. MORAN, Calcium release induced by interaction of opiate with smooth muscle cell microtubules. *Nature (Lond.)* 1972, 235 336-338.
- TRICK, M. A. M. O. FERNOLLET, P. MEYER, S. FERNANDEZ and P. FERNANDEZ, Angiotensin receptors smooth muscle cell microtubules. *Nature New Biol.* 1973, 243 55-58.
- THOM, L. and E. T. MACKENZIE, Amino mechanisms in the cerebral circulation. *Pharmacol. Rev* 1974 26 275-342.
- THOM, L., K. C. NELSON and CH. OSMAN, Influence of arterial tension and changes in sensitivity (wing) on induced contractions of pial arteries *in vivo*. *Arch. Int. Pharmacodyn. Ther* 1974, 208, 35-42.
- THOM, L. and C. OSMAN, Pharmacological characterization of adrenergic alpha and beta receptors regulating vascular responses of cerebral arteries *in vivo*. *Circulat. Res.* 1974 35 835-849.
- THOM, L. and CH. OSMAN, Pharmacological characterization of postsynaptic vasomotor receptors in the brain. In: *Neurogenic Control of the Brain Circulation* Ed. Ch. Osman and L. Edvinsson, Proc. 11th Symp. Witten-Greif, Vol. 30, Pergamon Press, Oxford and New York, 1977 p. 167-183.
- W. K. D. GANTY, T. HÖRTEL and P. BOLKE, Immunohistochemical evidence for the existence of angiotensin II-containing nerve terminals in the brain and spinal cord in the rat. *Neuroscience Letters* 1978 2 229-234.
- WILKINSON, W. P. O. SCHMID and M. I. PHILLIPS, Evidence for direct neuronal stimulation by intraventricular angiotensin II. *Brain Res* 1977 126 376-381.
- WILKINSON, L. N. A. T. R. SANCHEZ, P. A. SENEZ and J. SENEZ, Correlated electrical and mechanical responses of isolated pial arteries to some vasoactive drugs. *Stroke* 1979 Submitted for publication.
- WILKINSON, L. P. and D. PALAC, Characterization of the angiotensin receptor in guinea-pig aorta. *J. Pharmacol. exp. Ther* 1975 215 167-175.
- WILKINSON, L. H. and F. M. B. MULLER (Eds.), *Angiotensin*. Springer Verlag, Heidelberg 1974.
- WILKINSON, L. H. and J. W. MCCARTHY (Eds.), *Renal Hypertension*, Year Book Medical Publishers, Chicago, 1974.
- WILKINSON, L. P. H. A. KONTOS and J. L. H. THOMSON JR., Vasoconstrictor effect of angiotensin on pial arteries. *Stroke* 1978 9 457-459.
- WILKINSON, L. and CH. O. LOEW, Penetration of angiotensin II into the brain. *Neuropharmacol.* 1971 10 411-424.
- WILKINSON, L. B. O. Action of angiotensin on adrenergic nerve endings. *Federation Proc.* 1978, 37 199-202.

Potential clamp experiments on myelinated nerve fibres from alloxan diabetic rats

By

TOM BRISMAR

Diabetic neuropathy in man belongs to the group of neuropathies characterized by experimental demyelination (Thomas and Lascelles 1966). The mechanism for the development of this pathological change is not known. Investigations of rats with experimentally induced diabetes have shown that the animals get a reduced conduction velocity (Eliasson 1964, Sten and Thomas 1974) but the decreased conduction velocity was not related to structural changes as found in the human neuropathy (Sharma and Thomas 1974). The present paper describes changes in the nodal function that were observed in potential clamp experiments on single myelinated nerve fibres isolated from the alloxan diabetic rat.

Diabetes was induced by a intraperitoneal injection of a buffered solution of alloxan monohydrate (25-150 mg/kg b.w.t.). The animals were maintained on commercial standardized pelleted rat diet and water *ad libitum*. Large single myelinated nerve fibres (10-1 μ m) were isolated from the sciatic nerve of diabetic rats (Sprague-Dawley 30 g). Nerves were taken at the onset of weight loss and development of cataracts.

7 months after the induction of diabetes. The method of potential clamp of single myelinated nerve fibres was similar to that developed by Dodge and Frankenhaeuser (1958) and has been described in a investigation of normal rat myelinated nerve fibres (Brismar 1978). The experiments were performed at about 25°C and the isolated nerve was kept in oxygenated Ringer solution (147 mM NaCl, 5.9 mM KCl, 3.1 mM CaCl₂ and 5.0 mM Trizma, pH 7.4 at 25°C). The isolated single fibre was mounted in a recording chamber and connected to the feedback amplifier system which was used firstly to record the membrane potential and then to record the membrane currents associated with step changes in the membrane potential.

Fig. 1 shows records of the action potential in a fibre from a diabetic rat. The action potential was followed by a prolonged afterdepolarisation not observed in normal fibres.

The nodal membrane potential was clamped and recordings were made of the membrane current associated with step changes in membrane potential. Plots of the initial current (I_{Na}) vs membrane potential (U) curve in diabetic rat fibres indicated a similar dependence on potential of the Na permeability mechanism as in the normal fibre.

Large differences were found in the K permeability properties between diabetic and normal rat fibres. The delayed outward current during positive potential steps was large in the diabetic rat nerve (Fig. 2) and small in normal fibres (Brismar 1978). Also in the diabetic rat fibre the repolarisation after the positive pulse was associated with a large transient current tail which decreased with a slow exponential time course. These findings were consistent with a large delayed potential dependent increase in the K permeability and a high extranodal [K]. Fig. 3 shows the effect of pulse duration on the amplitude of the delayed outward current and on the current tail at repolarisation. Several current transients

Key words: Potential clamp, myelinated nerve diabetes, neuropathy

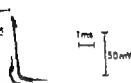


Fig. 1

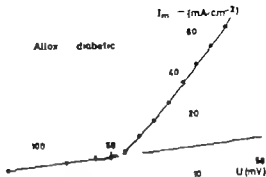


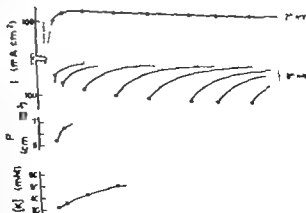
Fig. 2

Membrane action potentials and subthreshold responses in alloxan diabetic rat nerve fibres. Stimulus (saw) repeated at frequency of 1 Hz. Temp 26°C.

2. Steady state current-voltage relation in alloxan diabetic rat nerve fibre. Smooth curve fitted to whole. Straight line indicates leak current. Temp 26°C.

delayed with pulses of various duration have been superimposed. U was 20 mV during pulse and at the resting level (-80 mV) before and after. The delayed outward current decayed rapidly after the initial Na current and decreased slightly thereafter whereas the initial associated with repolarisation gradually increased in size with lengthening of pulse.

These results were analysed on the assumptions that (1) the delayed currents and the initial were carried by K⁺ (2) that the instantaneous K permeability (P_K) after a step in U is equal to the P immediately before the step, (3) that the relation between P and P satisfied the constant field equation (Hodgkin and Katz 1949; Dodge and Nicholls 1959) and (4) that the $[K]_i$ was 153 mM. On the basis of these assumptions, P_K and $[K]_o$ (extracellular [K]) were calculated from the measured outward I_K and inward I_{Na} associated with various pulse durations. Corresponding values of I_K , P_K and $[K]_o$ at each time are indicated by symbols in Fig. 3. The calculated change in P is pulse



3. Top: Outward I_K and inward I_{Na} in alloxan diabetic nerve fibres demonstrated by superimposed records of membrane associated with pulses of various duration. Symbols refer to values of experimental for the calculation of P and $[K]_o$ (extracellular [K]). Middle and bottom: P_K and $[K]_o$ calculated from I as indicated in text. Smooth curves are fitted to symbols.

duration showed an S-shaped increase towards a ceiling level (P_K $7.0 \text{ cm s}^{-1} 10^{-9}$) which was maintained during the pulse. This value is considerably higher than found in normal rats ($0.2 \text{ cm s}^{-1} 10^{-8}$ Brismar 1978). The calculations further indicated a large increase in $[K]_i$ during the positive potential steps. This finding may explain the high value of I_{K1} vs U curve in Fig. 2 and the presence of an afterdepolarisation (Fig. 1).

A large increase in the amplitude of the delayed current was found in 3 alloxan diabetic rats (the calculated P_K was 2.6, 4.0 and $7.0 \text{ cm s}^{-1} 10^{-9}$ respectively) and a moderate increase compared to the ordinary very small delayed currents was found in other alloxan diabetic rats. At present these changes can not be attributed with certainty to the diabetic condition as such as alloxan has toxic effects on other organs (e.g. kidneys), which may have contributed. It was concluded that (i) the normally low delayed P_K can, under certain (pathological) conditions, increase to very large values, (ii) a diffusion barrier hampers the K^+ transport from the extranodal space, (iii) due to the accumulation of K^+ , action potentials were followed by an afterdepolarisation which may inactivate the P_{Na} mechanism and thereby decrease the excitability of the nerve.

The present findings may be explained by the following hypothesis. Large delayed K^+ currents may have passed through the nodal membrane of normal fibres but were not found at potential clamp because of a shunt through the Schwann cell. The mechanism for this postulated K^+ current shunt in the normal rat fibre would thus be similar to the attenuation artifact at potential clamp of (frog) myelinated nerve fibres (Dodge and Frankenhaeuser 1958), but would be selective for the K^+ currents if the K^+ permeability channels are restricted to certain parts of the nodal area. Experimental evidence for such a K^+ pathway from axon to Schwann cell has been found in the squid (Vilegas 1972). If the Schwann cell-node contacts are disrupted in the alloxan diabetic rat nerve, as has been described in electron micrographs of early diaphoretic demyelination (Allt and Cavanagh 1969), the Schwann cell shunt would be lost. This could result in the large K^+ currents and the K^+ accumulation found in the alloxan diabetic rat.

This investigation was supported by the Swedish Medical Research Council (Project No. 14X-025) and the Foundations of Karolinska Institutet.

References

- ALLT, G. and J. B. CAVANAGH, Ultrastructural changes in the region of the node of Ranvier in the rat caused by diphtheria toxin. *Brain* 1969, 92, 459-468.
- BRISMAR, T., Potential clamp analysis of membrane currents in rat myelinated nerve fibres. 1978, Submitted.
- DODGE, F. A. and B. FRANKENHAUSER, Membrane currents in isolated frog nerve fibre under voltage clamp conditions. *J. Physiol. (Lond.)* 1958, 143, 76-90.
- DODGE, F. A. and B. FRANKENHAUSER, Sodium currents in the myelinated nerve fibre of *Triturus cristatus* investigated with the voltage clamp technique. *J. Physiol. (Lond.)* 1959, 143, 183-200.
- ELLAMON, S. G., Nerve conduction changes in experimental diabetes. *J. clin. Invest.* 1964, 43, 2333-2340.
- HODGKIN, A. L. and B. KATZ, The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol. (Lond.)* 1949, 108, 37-77.
- SHARMA, A. K. and P. K. THOMAS, Peripheral nerve structure and function in experimental diabetes. *J. Neurol. Sci.* 1974, 23, 1-15.
- THOMAS, P. K. and R. G. LANCELLI, The pathology of diabetic neuropathy. *Quart. J. Med.* 1966, 29, 489-509.
- VILEGAS, J., Axon-Schwann cell interaction in the squid nerve fibre. *J. Physiol. (Lond.)* 1972, 225, 75-90.

On the innervation of the ileal mucosa in the rat—a synapse

By

B. NEWSON, H. AHLMAN, A. DAHLSTRÖM, T. K. DAS GUPTA and L. M. NYHLIN

The classical concept of the innervation of the small intestine was that preganglionic excitatory cholinergic nerve fibres and postganglionic inhibitory adrenergic fibres synapse on acetylcholinesterase-positive intramural ganglion cells, thus modulating the activity of the final motor pathway to the smooth muscle cell. No intrinsic adrenergic neurons have been demonstrated in this part of the gastrointestinal tract, but extrinsic adrenergic fibres innervate directly the intramural blood vessels (cf Furness and Costa 1971). The mere existence of different adrenergic and peptidergic vagal pathways to the gut (cf Lundberg 1979) as well as parasympathetic and/or peptidergic intramural neurons (Burnstock 1972, Burnstock et al. 1977) has made the original dualistic concept of gut innervation far more complex.

A direct synaptic contact between nerve terminals and the gut epithelium has not yet been convincingly demonstrated at the ultrastructural level. The effects of adrenergic drugs on mucosal secretion have been explained to be mediated via the vascular bed, *i.e.* isoprenaline and noradrenaline both reduce mucosal blood flow and thereby inhibit gastric secretion. However isoprenaline inhibits acid secretion independently of mucosal blood flow, indicating separate adrenergic actions on vessels and secretory epithelium (Curwain & Hoban 1977). The purpose of the present study was to further investigate the possibility of a direct innervation of the gut epithelial cells.

Male rats 150 to 200 g were decapitated. Small mucosal specimens were prepared from terminal ileum. The specimens were fixed in 4% buffered glutaraldehyde, postfixated in 1% OsO₄, dehydrated and embedded in Araldite. Ultrathin sections (600 Å) stained with lead citrate and uranyl acetate were observed under a transmission electron microscope (MU 4 RCA).

The mucosal glands were basally surrounded by nerve processes, frequently enclosed in basement membrane cell sheaths. By tracing these axons in consecutive sections towards their terminal ends, a membrane-to-membrane contact between the nonterminal axon or the terminal axon and the gut epithelial cells was often found. This is well illustrated in Fig. 1 where the axons in a bundle of nonterminal axons make direct contacts with a capillary as well as the basal membrane of the enterocytes. In a few cases direct contact between nerve terminals and basal granulated cells with a true synaptic specialization was seen.

→ axon, nerve terminal, basal granulated cell, rat ileal mucosa, synapse

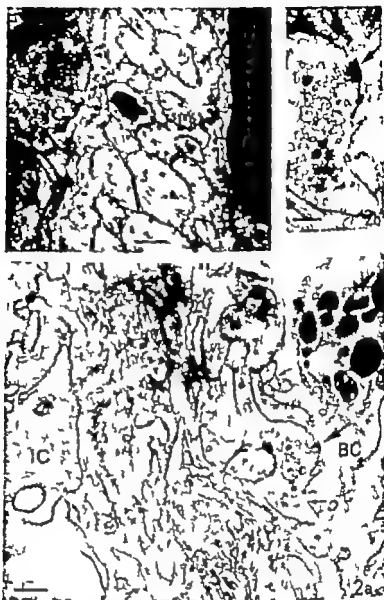


Fig. 1. From the rat ileal mucosa. A thick bundle of cross-sectioned nonterminal axons (A) in process between intestinalocytes (IC) to the left and a capillary to the right (RBC = red blood cell). The endothelium of the capillary and the basement membrane of IC can be seen in close apposition to the axons. Bar represents 185 nm.

Fig. 2a. Between two crypts of the rat ileal mucosa close to a basalgranulated cell (BC) to the right the nerve terminal varicosities (small arrows) can be seen. The lower one has a true synaptic contact with the BC (large arrow). Both large dense core vesicles and small round clear vesicles are present in the varicosity. Bar represents 400 nm.

Fig. 2b. Magnification of the synaptic area in Fig. 2a. Bar represents 200 nm.

(Fig. 2). The synapsing nerve terminal contained both large dense cored vesicles (about 80 nm) and small (40–50 nm) round empty vesicles, probably representing an adrenergic terminal. However since no particular treatment for the identification of adrenergic terminals was made this terminal may be either cholinergic, adrenergic or possibly a third type.

has earlier been stated that peripheral autonomic neurons can exert their action on target cells by transmitter release even without true synapses, provided that the distance of the "autonomic gap" does not exceed 100 nm (Burnstock and Costa 1975). Ultrastructural studies of the gut and pancreas have revealed such close relationships between different types of neurons and various endocrine cells, e.g. islet cells (Kobayashi and Fujita 1969), chromaffin cells (Lundberg et al 1978) and basalgranulated cells of the fetal enteron (Osaka and Kobayashi 1976). However, in this study we have observed a true synapse between an autonomic nerve terminal and a basalgranulated epithelial cell. This raises the possibility that such contacts may occur also elsewhere in the gastrointestinal tract, not only in the case of neuroendocrine complexes, but also between nerve terminals and other types of target cells. The presence of synapses on gut endocrine cells may indicate a nervous control of the release of secretory products from these cells (cf Ahlman 1976). From the present study it also seems evident that axons from the same axonal bundle can directly innervate both the gut epithelium and its vasculature.

This study was supported by grants from the Veterans Administration West Side Hospital and the C. T. Casper Foundation and from the Swedish Medical Research Council (17X 5220, 14X 2207 04P-4173).

References

- ALLEN, H. Fluorescence histochemical studies on serotonin in the small intestine and the influence of vagal nerve stimulation. *Acta physiol. scand.* 1976. Suppl. 437.
- BLUM, G. Parasympathetic nerves. *Pharmacol. Rev.* 1977. 31 509-531.
- BLUM, G. and M. COSTA. Adrenergic neurotransmitter transmission. I. *Adrenergic neurones*. Chapman and Hall, London 1975.
- BLUM, G. P. and P. HOLTER. The effects of isoprenaline and noradrenaline on pentagastrin-stimulated gastric acid secretion and splanchnic blood flow in the dog. *Acta J. Pharmacol.* 1972. 46 225-231.
- BLUM, G. P. and M. COSTA. Morphology and distribution of intramural adrenergic neurones in the small intestine of the guinea-pig. *Z. Zellforsch.* 1971. 120. 345-363.
- BLUM, G. P. and T. FLATA. Fine structure of mast cells and enteric pancreatic islets with special reference to D cells and nervous elements. *Z. Zellforsch.* 1969. 100 340-363.
- BLUM, G. P., T. HÖGSTRÖM, G. MÖLLER, G. PETERSSON, J. KERNER, H. AHLMAN, R. EDIN, A. DANTZON, L. TERNER, and S. BÄCK. Substance P, VIP and enkephalin-like immunoreactivity in the human small intestine. *Gastroenterology* 1979. (In press).
- BLUM, G. P., A. DANTZON, A. BYLÖCK, H. AHLMAN, G. PETERSSON, L. LARSSON, H. A. HANSSON and J. KERNER. Ultrastructural evidence for an innervation of epithelial enterochromaffin cells in the human small intestine. *Acta physiol. scand.* 1978. 104 3-12.
- BLUM, G. P. and S. LARSSON. Duodenal basalgranulated cells in the human ileum with special reference to their relationship to nervous elements. I. *Endocrine gut and pancreas*. Ed T. Fujita. Elsevier 1976.
- BLUM, G. P., J. M. FUKUI, S. R. BLOOM. The enteric gut hormones. Cellular sources, physiology and clinical aspects. *Gastroenterology* 1977. 72 744-761.

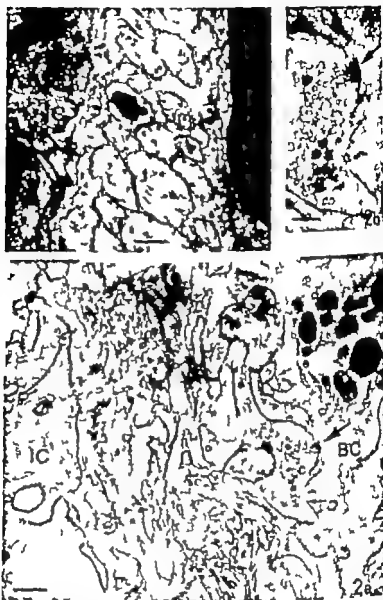


Fig. 1 From the rat ileal mucosa. A thick bundle of cross-sectioned nonterminal axons is present between enterocytes (IC) to the left and capillary to the right (RBC—red blood cell). The endothelium of the capillary and the basement membrane of IC can be seen in close apposition to the axons. Bar represents 185 nm.

Fig. 2 a. Between two crypts of the rat ileal mucosa close to a basal ganglion cell (BC) to the right and nerve terminal varicosities (small arrows) can be seen. The lower one has a true synaptic contact with the BC (large arrow). Both large dense core vesicles and small round clear vesicles are present in the nerve terminal. Bar represents 400 nm.

Fig. 2 b. Magnification of the synaptic area in Fig. 2 a. Bar represents 200 nm.

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References

- ALLEN, H. Fluorescence histochemical studies on serotonin in the small intestine and the influence of vagal nerve stimulation. *Acta physiol. scand.* 1976. Suppl. 437.
- BOCK, O. Pharmacologic review. *Pharmazie* 1972. 24 509-581.
- BOCK, O. and M. COSTA. Adrenergic neuroeffector transmission. In *Adrenergic nervous system*. Chaptman and L. L. London 1975.
- BOCK, O. P. and P. HOLTEN. The effects of isoprenaline and noradrenaline on postgastric-stimulated gastric acid secretion and mucosal blood flow in the dog. *Brit. J. Pharmacol.* 1972. 45. 223-231.
- BOCK, O. P. and M. COSTA. Morphology and distribution of intrinsic adrenergic neurons in the fundal colon of the guinea-pig. *Z. Zellforsch.* 1971. 120. 346-363.
- BOCK, O. P. and T. FLATA. Fine structure of submucosal and islet pancreatic islets. 10th special symposium on D cells and nervous elements. *Z. Zellforsch.* 1969. 100. 340-343.
- BOCK, O. P., T. HÖKfelt, G. NERLSON, O. PETERSSON, J. KREWENTER, H. AHLMAN, R. EDVÄ, A. DAMEL, L. TERNER and S. SÄND. Substance P- VIP- and enkephalin-like immunoreactivity in the human gut. *Gastroenterology* 1979 (in press).
- BOCK, O. P., A. DAMELSTRÖM, A. BYLÖCK, H. AHLMAN, O. PETERSSON, I. LARSSON, H.-A. HANSSON and XI. INTY. Ultrastructural evidence for an innervation of epithelial enterochromaffin cells in the human gut. *Acta physiol. scand.* 1978. 104. 3-12.
- BOCK, O. P. and S. KÖNIG. Duodenal basalgranulated cells in the human fetus. 10th special symposium on D cells and nervous elements. In: *Endocrine gut and pancreas*. Ed. T. Fujita. Elsevier 1976.
- BOCK, O. P., J. M. FULAK, S. R. BLOOM. The secret gut hormones. Cellular sources, physiology and clinical aspects. *Gastroenterology* 1977. 72. 744-761.



Fig. 1 From the rat ileal mucosa. A thick bundle of crosssectioned nonterminal axons is present between interstitial cells (IC) to the left and capillary to the right (RBC—red blood cell). The radiothick axon of a capillary and the basement membrane of IC can be seen in close apposition to the axons. Bar represents 185 nm.

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Fig. 2 b. Magnification of the synaptic area in Fig. 2 a. Bar represents 700 nm.

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References

- ALM, K., Fluorescence histochemical studies on serotonin in the small intestine and the influence of vagal nerve stimulation. *Acta physiol. scand.* 1976, Suppl. 437.
- BLUM, G. Parasympathetic nerves. *Pharmacol. Rev.* 1972, 24, 309-381.
- BURNSTOCK, G. and M. COSTA, Adrenergic neuroeffector transmission. In *Adrenergic nervous system*, Chapman and L. London 1975.
- CHEN, S. P. and P. HOLTER, The effects of norepinephrine and noradrenaline on postgastric-stimulated gastric acid secretion and mucosal blood flow in the dog. *Brit. J. Pharmacol.* 1972, 46, 225-231.
- CHEN, J. B. and M. COSTA, Morphology and distribution of autonomic adrenergic nerves in the small intestine of the guinea-pig. *Z. Zellforsch.* 1971, 120, 344-363.
- CHEN, S. and T. FLÖTTA, Fine structure of mucosal and submucosal ganglion cells in the small intestine of the guinea pig and nervous elements. *Z. Zellforsch.* 1969, 100, 340-363.
- CHEN, S. T. HÖRTEL, G. NILSSON, G. PETERSSON, J. KREYTER, H. AHLMAN, R. EDRE, A. DANIELSSON, L. TERNER and S. SÄÖ, Serotonin P. VIP and enkephalin-like immunoreactivity in the human small intestine. *Gastroenterology* 1979 (in press).
- CHEN, S. T., A. DANIELSSON, A. BYLÖCK, H. AHLMAN, G. PETERSSON, I. LARSSON, H.-A. FLYNN and J. KREYTER, Ultrastructural evidence for an innervation of epithelial endochromaffin cells in the human small intestine. *Acta physiol. scand.* 1978, 104, 3-12.
- CHEN, S. T. and S. KONG, Duodenal basalgranulated cells in the human fetus with special reference to their relationship to nervous elements. In: *Endocrine gut and pancreas*, Ed. T. Fajta, Elsevier 1976.
- CHEN, S. T., A. G. E. J. M. POLAK, S. R. BLUM, The secretory gut hormones. Cellular sources, physiology and clinical aspects. *Gastroenterology* 1977, 72, 744-761.

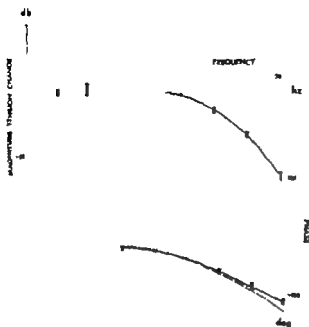


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Fig. 2b. Magnification of the synaptic area. Fig. 2a. Bar represents 200 nm.

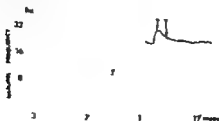
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Modulation of the isometric tension of a typical unit as a function of modulating frequency. The slope of the graph describes the magnitude of tension changes while the bottom describes the phase shift. The solid line is tension and points in stimulus rate. The solid line is drawn through the means of individual data with the bars representing the variation in six consecutive cycles. The thin dashed line shows the magnitude and phase of a linear critically damped second order model with a natural frequency of 7.8 Hz. The thick dashed line in phase represents the effect of an added 7 ms delay to the phase model.

2. Beyond 2 Hz there is a rapid and progressive decrease in the tension change that is for the same change in stimulus rate. The magnitude was graphically fitted to a second order system which in the case of this particular unit had a natural frequency of 7.8 Hz. This parameter describes the point at which straight lines fitted to low and high frequency data points intersect.

3. The phase lag or delay with which tension changes follow changes in stimulus rate is plotted in the lower portion of Fig. 1. The phase predicted by a second order system is



2. The natural frequencies of 12 units with twitch responses in relation to their twitch properties. The time to peak tension, \square half-decay time, \bullet average of rise time and half decay time. Inset; typical response.

Dynamics of isometric tension in single motor units in the inferior oblique muscle of the cat

By

T. VILIS and G. LENNERSTRAND

The extraocular muscles of the cat are unique in that the muscles are composed of three distinct types of motor units: singly innervated units (SI), multiply innervated conducting units (MIC) and multiply innervated nonconducting units (MINC) (Lennérstrand 1974). Previously the isometric mechanical properties of these units have been categorized in terms of twitch response, fusion frequency and rate of tension rise (Lennérstrand 1974). An additional method of analysing their isometric mechanical properties is the frequency response. This describes the muscle fiber's ability to generate both increases and decreases in isometric tension in response to sinusoidally increasing and decreasing stimulus rate. We found that all three types of units could most simply be characterized in terms of a linear overdamped second order system similar to that used to describe fibers in the soleus (Mannard & Stein 1973).

Experiments were done on adult cats anesthetized with pentobarbital. Preparation of functionally intact motor units in the inferior oblique muscle, method of electrical stimulation, and method of recording tension is described in detail by Lennérstrand (1974).

The term stimulus rate or simply rate, will be used in this paper to describe the number of electrical pulses per second delivered to the muscle nerve. The modulating frequency will describe the period length in time, between two peaks in the sinusoidal modulation of the stimulus rate. The difference between the minimum and maximum rate will be called the modulation depth and their average the carrier or mean rate.

Fourier analysis was performed on muscle tension using a PDP11 computer to obtain (1) an estimate of the magnitude of tension change produced in response to changes in stimulus rate and (2) the delay or phase lag with which changes in tension followed changes in stimulus rate.

14 single motor units were fully studied. These, on the basis of their isometric tension properties (Lennérstrand 1974) were subdivided into three types. 9 units had rise contraction times shorter than 7 ms and fusion frequencies greater than 700 Hz (range of SI units). 3 units showed contraction times above 5 ms and had fusion frequencies between 90 and 175 Hz (range of MIC units). The remaining 2 units did not exhibit twitch response while tetanic tension fused at stimulus rates below 75 Hz (properties of MINC units).

The frequency response typical of all units, is shown in Fig. 1. The amplitude of the change in isometric tension is fairly constant for modulating frequencies between 0.25 and 10 Hz.

Anaphylatoxin-induced shock and two patterns of anaphylactic shock: Hemodynamics and mediators*

By

K. PAVEK, PRISCILLA J. PIPER and G. SWEDEGÅRD

Received 4 July 1978

Abstract

Dr K., P. J. PIPER and G. SWEDEGÅRD. *Anaphylatoxin-induced shock and two patterns of anaphylactic shock. Hemodynamics and mediators*, Acta physiol. scand. 1979 105 393-403

Two different cardiorespiratory reactions were identified in two types of anaphylactic shock and in AT (anaphylatoxin)-induced shock. All three types had in common: portal blood pooling with consequent decrease in the venous return, cardiac output, and arterial pressure. In anaphylaxis (A) of the IgE type, a low titer of hemagglutinating antibodies, the latent period was 65 s and heart and lung function was unchanged. In the second type of high titer the latency was 19 s and pulmonary hypertension and decreased heart contractility occurred. After AT injection pulmonary hypertension appeared and unchanged heart function. Tachyphylaxis, but not cross-over tachyphylaxis against anaphylactic agent and AT is observed in dogs and isolated guinea pig lungs. AT induced transient release and a prolonged release of histamine, prostaglandins (PGs), and thromboxane A_2 and increases liver guinea pig lungs. SRS-A was released only in a. Indomethacin reduced AT-induced release of PGs in guinea pig lungs and AT-induced hypertension in the dog though it did not prevent the fall in cardiac output. These model studies suggest that different patterns of clinical shock occur depending on the type of antibodies and/or mediators involved.

*and C5a-anaphylatoxin, histamine, prostaglandins, SRS-A, chemical forms of anaphylaxis

Very little is known about the hemodynamics of the cardiovascular shock which is occasionally prominent in human systemic anaphylaxis (Kelly and Patterson 1974). In a key model of aggregate anaphylaxis the involvement of the heart, the pulmonary and systemic circulation was studied (Pavek 1977) but it was also interesting to establish whether the cardiovascular shock has a single, uniform pattern or whether there are several patterns of cardiovascular involvements depending perhaps on the type of antibodies and/or type of mediators involved. In this study we were able to identify different patterns of cardiovascular shock in two dog models of anaphylaxis, and in shock induced by C5a hog anaphylatoxin (AT), a product of complement activation. We were also able to identify

A shortened form of this report appeared as poster at the 1975 European Immunology Meeting, London.

shown as a thin dashed line. If, in addition, one introduces a delay of 7 ms which could be accounted for by delays introduced by the sinusoidal variation of the stimulus rate and by conduction and synaptic delays, the theoretical curve, thick dashed line, provides a good fit to the experimental data.

The range of natural frequencies for units exhibiting twitch tension (SI units and MLC units) is given in Fig. 2. It shows not surprisingly that units that have slow contraction times generally exhibit low natural frequencies. The lowest natural frequencies, 4 Hz, were exhibited by the two units which did not show a twitch tension (not shown in Fig. 2). Units which exhibited twitch tension responded faster; that is, their natural frequencies increased if the magnitude of the modulation depth was increased. In the two units with no twitch response increase in the modulation depth produced a decrease in the natural frequency.

It has been suggested (Mannard & Stein 1973) that close fit of the muscle frequency response data to a second order system underlies the fact that there are predominantly two rate-limiting processes in the modulation of isometric muscle tension, the process of binding between actin and myosin and the process involved in the decay of the active state. The fact that all three types of motor units in the extraocular muscles have frequency responses which fit the same model suggests that modulation of tension here is also limited by the same process. It is not unexpected that the natural frequencies, especially of units thought to be SI, are generally higher than those found in the soleus or gastrocnemius muscles (Mannard & Stein 1973; Partridge 1965). It was noted however in eleven of the twelve units that exhibited twitch tensions, that decay of tension was slower than rise (Fig. 3) suggesting that the process preventing the natural frequencies from being higher still was the decay of the active state.

This work was supported by grants from the Medical Research Council of Canada and from the Swedish Medical Research Council (no 4751 and 3875).

References

- MANNARD, A. and R. B. STEIN, Determination of the frequency response of isometric soleus muscle in the cat using random nerve stimulation. *J. Physiol. (Lond.)* 1973, 229, 275-296.
- PARTIDGE, L. D. Modifications of neural output signals by muscles. Frequency response study. *appl. Physiol.* 1965, 20, 150-158.
- LENNERSTRAND, G. Motor units in eye muscles. In: G. Lennerstrand and P. Bach-y-Rita (Eds) *and Mechanisms of Ocular Motility and their Clinical Implications*. 1973, Oxford, Pergamon Press, pp. 13-143.
- LENNERSTRAND, G. Electrical activity and isometric tension in motor units of the cat's inferior oblique muscle. *Acta physiol. scand.* 1974, 91, 458-475.

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tachypnea and recharged heart function. Tachypylaxis, but not cross-over tachypylaxis against
anaphylactic agent and AT was observed in dogs and isolated guinea pig lungs. AT induced trans-
ient and prolonged release of histamine, prostaglandins (PGs), and thromboxane A_2 and
prostaglandin from guinea pig lungs. SRS-A was released only in a. Iodocetane inhibited AT-induced
release of PGs in guinea pig lungs and AT-induced hypotension in the dog though it did not prevent the
decrease in cardiac output. These model studies suggest that different patterns of clinical shock can occur depending
on the type of antibodies and/or mediators involved.

*with Clo-anaphylatoxin, histamine, prostaglandins, SRS-A, clinical forms of anaphylaxis

Little is known about the hemodynamics of the cardiovascular shock which is occa-
sionally prominent in human systemic anaphylaxis (Kelly and Patterson 1974). In a
recent model of aggregate anaphylaxis the involvement of the heart, the pulmonary and
systemic circulation was studied (Pavze 1977) but it was also interesting to establish
whether the cardiovascular shock has a single, uniform pattern or whether there are several
patterns of cardiovascular involvements depending perhaps on the type of antibodies and/or
type of mediators involved. In this study we were able to identify different patterns of
cardiovascular shock in two dog models of anaphylaxis, and in shock induced by C5a hog
anaphylatoxin (AT), a product of complement activation. We were also able to identify

As abstracted form of this report appeared as poster at the 1975 European Immunology Meeting,
München.

differences in mediators released during anaphylaxis after injection of AT into sensitized guinea pig lungs. The release of mediators after injection of AT was also studied in the intact dog.

Methods

Isolated perfused guinea pig lungs

The lungs were removed from male guinea pigs (300–400 g) and perfused with Krebs-Henseleit solution via the pulmonary artery. Lungs from either normal or ovalbumin pre-sensitized guinea pigs were used. The effluent from the lungs superfused a series of assay tissues to detect the mediators of anaphylaxis. These were strips of cat terminal ileum (CTI) to detect histamine; rabbit aorta (RA) to detect rabbit aorta contracting substance (RCS) now known to be a mixture of thromboxane A_2 and prostaglandins G_2 and H_2 (Hamberg et al. 1975); longitudinal smooth muscle of guinea pig ileum to detect slow-reacting substance of anaphylaxis (SRS-A) and a rat stomach strip (RSS) and colon (RC) and chick rectum (CR) to detect prostaglandins (PGs). All assay tissues except CTI were made more specific for RCS, SRS-A, and PGs by continuous infusion of antagonists to acetylcholine, 5-hydroxytryptamine, histamine, and catecholamines (Piper and Vane, 1969).

Identification of prostaglandins

Effluent from lungs was collected either directly from the cut heart or after superfusion over the tissues. Samples were collected immediately after infusion of AT into the pulmonary artery, and also from untreated control lungs. The effluent was acidified to pH 3 and extracted twice with ethyl acetate evaporated to dryness, and subjected to thin layer chromatography in the AI system (Gries and Smeyers-Nebe, 1964).

Detection of substance released into the circulation of the dog

Dogs were anesthetized with pentobarbital sodium 30 mg/kg and prepared for use in the blood inlet organ technique (Vane 1969). An endotracheal tube was inserted to facilitate artificial respiration; the administration of heparin 1 000 u/kg b.wt., blood was pumped at 10 ml/min, passed through a dialyzer machine (Cofler et al. 1973), and returned to the opposite femoral vein. The substances present in the dialysate were estimated by the assay tissues as previously described.

Hemodynamics in anaphylaxis and anaphylaxis-induced shock

In the A group 5 dogs were sensitized to horse serum by an i.v. dose of 20 ml followed by 3 i.m. injections of 5 ml over the following 3 days. After 3 to 4 weeks, a shock was elicited by challenge with right atrial injection of 10 ml horse serum.

In the B group, 7 dogs were sensitized by 20 ml of horse serum given i.v. and later at 2–3 weeks interval with 1 + 1 ml of horse serum with Freund's adjuvant given i.m. In the first booster dose, complete Freund's adjuvant was used, while in the second, incomplete Freund's adjuvant was used since repetitive administration of complete adjuvant was not tolerated locally. The dogs were challenged by right atrial injection of 10 ml horse serum one week after the last booster dose. In some of the sensitized dogs, antiserum and antibody titres were measured using passive hemagglutination. Blood sample was taken before the challenge.

In the third group (C group) of 12 dogs AT was injected. AT (CSa) was isolated from yeast-activated horse serum using the described procedure (Leflander et al. 1972). 24 mg of AT were obtained from 36 horse serum. Purity of AT was tested by polyacrylamide electrophoresis and 1 strong and 1 weak band was detected. Two dogs received iodoethane premedication, 3 mg/kg given i.v. 10 min before the injection of AT.

Shock experiments in dogs were performed under pentobarbital anesthesia. Respiration was spontaneous and secured by cuffed tracheal tube. After dissection of appropriate vessels, catheters were placed into desired sites partly under X-ray control. Vascular pressures in the portal vein, right and left atria, pulmonary artery and distal aorta were measured using catheters and Statham transducers. Cardiac output was estimated by left-sided thermodilution technique. Curves were detected in the aortic arch and a digital value of cardiac output appeared shortly after the injection of cold saline on display of a cardiac output computer (Pavek 1974). Pressures and thermodilution curves were recorded on a UV recorder (Atlas Copco). In some of the experiments ECO was also recorded and hemoglobin concentration measured. O₂ saturation was measured in blood sampled from both systemic and pulmonary arteries (CO-Oximeter Instrumentation Laboratories).

TABLE I. CS-anaphylatoxin-induced release of mediators from isolated perfused guinea pig lungs.

LA—into pulmonary artery DIR—directly to assay tissues.

Duration of infusion	Indomethacin 1 µg/ml infusion	Histamine µg/ml	RCS	PGs µg/ml		Increase in Pulmonary artery pressure, mmHg	Comments
				E _a	F _{5t}		
2 min	—	300	+	10	5		(+)
2 min	DIR	300	+	10	10-20		
2 min	LA	300	—	<2	0		
2 min	DIR	300	+	20	>20		
2 min	DIR	>300	+	20	30		
2 min	LA	—	—	0			
2 min	DIR	300	+	10	<10	50-80	
2 min	DIR	1000	+	20	<20	30-50	
2 min	LA	100	—	1	0	35-55	
2 min	DIR	1000	+	20	20	Prolonged rise of about 100	
6 min	DIR	1000	+	10	20	Very small	(+)

2 min effect DIR.

2 min DIR. No effect. Only histamine release slightly longer than with 2 min LA infusion.

Results

Mediator release from isolated lungs

Piper (1972) showed that raw anaphylatoxin from rat serum released histamine-like and prostaglandin-like material from isolated perfused lungs. Our results with pure hog CS-A-T confirm and extend these findings (Table I). AT had no direct effects on the assay tissues but when AT 10 µg was injected or 1-2 µg/ml infused for 2 min into the pulmonary artery of guinea pig isolated lungs, all assay tissues except guinea pig ileum contracted (Table I). These results showed the release of histamine, RCS and a PG-like substance (Table I). In experiments where AT was infused into lungs from sensitized guinea pigs, challenge of the lungs produced similar contractions of the assay tissues. The amounts of histamine released, as estimated by contractions of CTI, were 0.5-1 µg/ml compared with 0.5-2 µg/ml released during anaphylactic shock, additionally SRS-A was released in anaphylaxis (Piper and Vane 1969). Contractions of RSS, CR, RC indicated that 5-20 µg/ml of prostaglandin-like material was released (assayed in terms of PGE₂ and PGE₁ 20-100 µg/ml (assayed as PG E₂) were released in anaphylaxis (Piper and Vane 1969). Extraction and thinlayer chromatography of effluent from lungs during infusion of AT showed that PGs of E and F-type were released.

It was not possible to quantitate the release of RCS owing to the instability of the substance but AT released an RCS which was indistinguishable from that released by anaphylaxis. When AT was infused over 6-10 min, the release of histamine, RCS and PGs was not prolonged. This shows that AT belongs to the group of substances which cause a short-acting release of histamine, PGs and RCS (Piper 1974).

In 3 experiments indomethacin 1 µg/ml was infused into the lungs before AT the subsequent release of RCS was abolished and that of PGs either abolished or very much reduced.

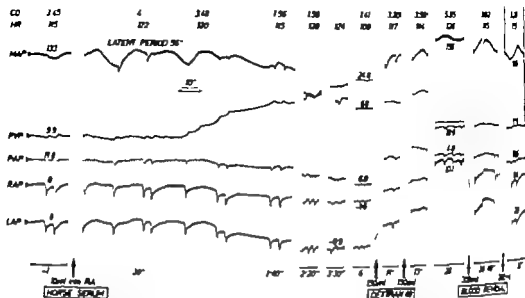


Fig. 1 Type A anaphylactic shock, dog no. 49. CO—cardiac output, HR—heart rate, pressure: MT—arterial, PVP—port I venous, PAP—pulmonary artery, RAP—right atrial, LAP—left atrial.

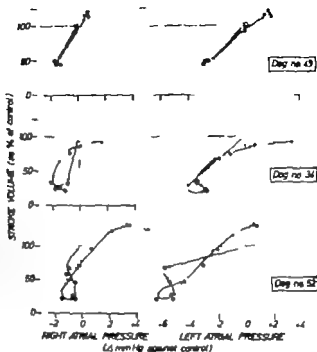
Substances released into the circulation

In 5 dogs $10 \mu\text{g}$ AT/kg was slowly injected i.v. There was a rapid fall in arterial blood pressure (40 mmHg) and a slow recovery over 10 min. Sometimes there was a small rise of pressure at the maximum point of hypotension. Following AT injection substantial contracting assay tissues entered the circulation. CTI, RSS, CR and RC contracted. CR and RC were blocked intraluminally with combined antagonists and their contraction showed the release of PG-like material. This release declined slowly as the blood pressure recovered. The contractions of RSS, CR and RC were almost matched by infusions of PGs $2.5 \mu\text{g}$ infused directly over the assay tissues. The contraction of CTI suggested the release of histamine or kinins. Rabbit aorta showed variable responses and no definite evidence of RCS release.

In 3 experiments where arterial blood was dialysed after AT injection, substances contracting assay tissues, like histamine and PGs, reached the assay tissues. Similar contractions of RSS, CR, RC were produced by dialysis of blood containing PGE₂, $5 \mu\text{g}/\text{ml}$. Dialysis of blood containing histamine $2 \mu\text{g}/\text{ml}$ caused a contraction of CTI similar to that seen after injection of AT. The contraction of CTI following injection of AT was terminated by superfusion of CTI with mepyramine. This strongly suggested that the contraction was due to histamine. Although kinins contract CTI they are not dialysed out of blood (Collins *et al.* 1973).

Hemodynamics

Anaphylactic shock type A This type of shock occurred in dogs sensitized only with hypodermic serum. A typical sequence of the cardiovascular events is shown in Fig. 1. The arterial changes in Fig. 4. After a challenging dose of serum there was a latent period of 60 s.



2. Ventricular function curves for A (dog no. 49) and type B (dog no. 34 and 52) shock. Curves show values after the challenge. Points are values during shock after increase in pressure by dextran.

\pm S.D.) with a range from 40 to 105 s. This period was measured as time between appearance of the cold bolus of horse serum in the aortic root as recorded by the transducer and the first distinct change in the portal venous pressure. The first detectable change was a transient increase of cardiac output. A pronounced increase in the portal venous pressure was accompanied by a small and transient (< 1 min) increase in the pulmonary artery pressure. Later the pulmonary artery pressure decreased below control level. About 10 s after the beginning of the portal hypertension a decrease in both atrial pressures and cardiac output appeared followed by a drop in the systemic arterial pressure and bradycardia. The oxygen saturation of arterial blood was unchanged, the arterio-venous difference in O₂ content widened as cardiac output decreased and in the first phase the hemoglobin concentration did not indicate a consistent trend towards hemoconcentration. No early changes in the respiratory frequency was observed with the exception of one case where a rapid breathing appeared (Fig. 6). However the respiratory frequency was always high during the developed shock.

In order to evaluate the pumping function of the heart during shock, atrial pressures were increased by repeated injections of dextran in increments of 50 ml. The resulting stroke volumes were plotted against the left atrial and the right atrial pressures (Fig. 2, dog no. 49). All points during the initial shock and after dextran-induced changes lay on or near a straight line which crosses the initial control value. This relation was present in all dogs and pointed against the presence of a depressed cardiac contractility in type A

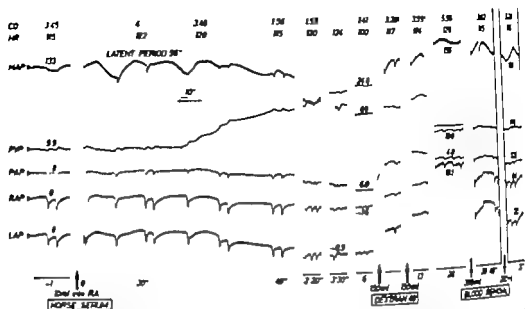


Fig. 1 Type A anaphylactic shock, dog no. 49. CO—cardiac output, HR—heart rate, pressure MAP—mean arterial, PVP—portal venous, PAP—pulmonary artery, RAP—right atrial, LAP—left atrial.

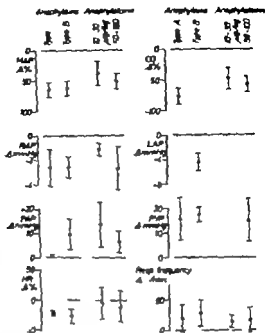
Substances released into the circulation

In 5 dogs $10 \mu\text{g}$ AT/kg was slowly injected i.v. There was a rapid fall in arterial blood pressure (40 mmHg) and a slow recovery over 10 min. Sometimes there was a small rise of pressure at the maximum point of hypotension. Following AT injection substantial contractions entered the circulation. CTI, RSS, CR and RC contracted. CR and RC were blocked intraluminally with combined antagonists and their contraction showed the release of PG-like material. This release declined slowly as the blood pressure recovered. The contractions of RSS, CR and RC were almost matched by infusions of PGs $2.5 \mu\text{g}$ infused directly over the assay tissues. The contraction of CTI suggested the release of histamine or kinins. Rabbit aorta showed variable responses and no definite evidence of RCS release.

In 3 expts. where arterial blood was dialysed after AT injection, substances contracted assay tissues, like histamine and PGs, reached the assay tissues. Similar contractions of RSS, CR, RC were produced by dialysis of blood containing PGE₂ $5 \mu\text{g}/\text{ml}$ dialysate blood containing histamine $2 \mu\text{g}/\text{ml}$ caused a contraction of CTI similar to that seen after injection of AT. The contraction of CTI following injection of AT was terminated by perfusion of CTI with mepyramine. This strongly suggested that the contraction was due to histamine. Although kinins contract CTI they are not dialysed out of blood (Cohn *et al.* 1973).

Hemodynamics

Anaphylactic shock type A This type of shock occurred in dogs sensitized only with bovine serum. A typical sequence of the cardiovascular events is shown in Fig. 1. The average changes in Fig. 4. After a challenging dose of serum there was a latent period of 68 ± 12 min.



4. Minimum cardiorespiratory changes were values \pm 95% confidence interval in 3 types of shock. Values \pm 200 were taken as after AT 50-100 μ g/kg; 50 mmHg in other groups. Number of points ≥ 3 except for LAP \pm 2, 3 in AT type PVP 3 after AT 50-100 μ g/kg.

Comparison of serum levels of hemagglutinating antibodies against horse serum showed that dogs sensitized with serum and Freund's adjuvant have a level of the order 200 times higher than dogs sensitized with serum alone.

4. *Effect of AT* AT was administered in 9 cases into the right atrium, in 2 cases into the left ventricle and in 1 case into a tributary of the portal vein. The ensuing reactions were similar. An estimation of the dose-response showed strong reactions in the dose range 100 μ g/kg (Fig. 4). The latent period was 17 ± 7 s and varied between 10 and 30 s.

After right atrial injections, first a pulmonary hypertension and some seconds later a systemic hypertension started. While the pulmonary reaction lasted about 5 min, the portal hypertension was sustained. The heart filling and cardiac output reacted similarly as in the anaphylaxis, heart rate varied and sinus rhythm was maintained. The respiratory frequency increased simultaneously with the pulmonary hypertension and at its height the tachypnea was a rule, directly proportional to the hypertension (Fig. 6).

After cessation of the decreased right atrial pressure by dextran or autologous blood, the cardiac output returned to its control level. Ventricular function curves showed that values of stroke volume versus right atrial pressure were near a line crossing the control value (Fig. 5); this testified that the cardiac contractility was not changed after AT injection.

The dogs premedicated by indomethacin did not show the usual hypotension after 10 μ g/kg. The cardiac output, however, was decreased by 30-40 per cent.

5. *Tachyphylaxis, cross-over tachyphylaxis.* In 3 dogs, after recovering from anaphylactic shock (type II) horse serum was injected again, but failed to induce measurable response.

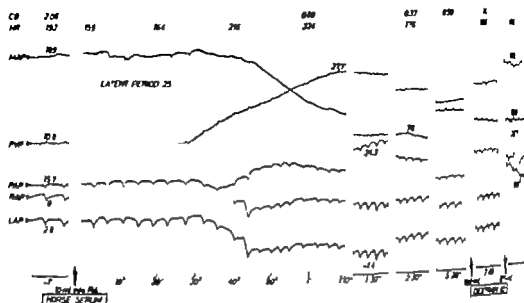


Fig. 3. Type B anaphylactic shock, dog no. 14

Evaluation of measurements during the shock where the low level of the right atrial pressure was corrected by dextran as nearly as possible to the baseline level, showed that at those times not only cardiac output but also pulmonary artery pressure returned to its control level. Also arterial pressure returned almost to the control level. Thus there was no active change in the pulmonary vascular resistance and only a variable and small decrease in the peripheral resistance.

Anaphylactic shock type B This type occurred in dogs sensitized with horse serum as Freund's adjuvant. The latent period was short, on average 19 ± 8 s and varied between 10 and 30 s. Similarly as in the type A initially portal hypertension appeared followed by a decrease in atrial pressures and later by a systemic arterial hypotension. At the low cardiac output there was regularly a sinus bradycardia (Fig. 4). A pronounced portal hypertension appeared early, reached maximum about 90 sec after challenge, and stayed for 5 min or more (Fig. 3). There was no consistent hemoconcentration or decrease in the saturation of arterial blood and the arteriovenous difference in O₂ content widened as cardiac output dropped. The rate of respiration initially varied in both directions, occasionally short apnea was observed. Since the shock was developed, tachypnea was a rule.

Construction of ventricular function curves (Fig. 2) showed signs of right heart depression in 6 out of 7 dogs, and of left heart depression in 5 out of 7 dogs. In 4 cases the myocardial depression lasted less than 5 min and in 2 cases it was present over the whole observation period, i.e. more than 15 min.

Evaluation of the selected measurements, where the lowered right atrial pressure was corrected by dextran closely to its baseline values, showed that the pulmonary artery pressure was consistently increased on the average by 75%. The mean arterial pressure remained consistently low on the average 17% under the control value.

Thus in type B shock there was a marked and active increase in the pulmonary vascular resistance.

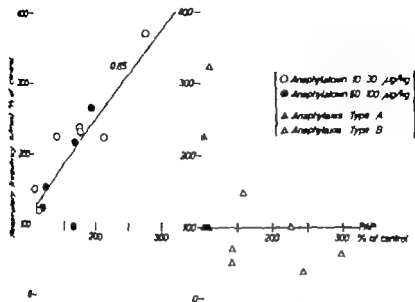


Fig. 2. Respiratory frequency at the highest level of pulmonary artery pressure in 3 types of shock.

The second of our findings is that one set of cardiorespiratory patterns was regularly found to be sustained over a short period with the anaphylactic agent only; somewhat different ones occurred in dogs sensitized over a longer period with the agent in Freund's adjuvant, again another set of changes was associated with the AT-induced shock. Our findings also indicate that different mechanisms were involved in the anaphylactic and AT-induced shocks. This suggestion is supported by the demonstration of a complete anaphylaxis to the anaphylactic agent and to AT in both isolated guinea pig lungs and in dogs—though under conditions of tachyphylaxis to one of the two agents, a full anaphylactic response could be sustained by the other agent. The presently observed effects of AT on the pulmonary and systemic circulations are similar to findings of Bodanszky in the guinea pig and cat. Bodanszky concluded that these effects of AT are direct and are mediated by released histamine or prostaglandins. In our experiments histamine and prostaglandins were released after both the anaphylactic agent and AT. SRS, however, is released only in the anaphylaxis. Since AT readily released histamine and prostaglandins when injected into the pulmonary circulation, it may act both directly and via these mediators. The fact that indomethacin inhibited the AT-induced hypotension suggests that some product(s) of metabolism of arachidonic acid by cyclo-oxygenase was involved in this response. Further it appears that the titer of antibodies and the associated state of hypersensitivity to the mediator and receptor levels are important determinants of the cardiorespiratory response in the anaphylactic shock. Dogs with high titers of hemagglutinating antibodies have a more severe shock, a strong tendency towards a major and sustained elevation of the pulmonary vascular resistance and decreased cardiac contractility; such changes were not observed in

ANAPHYLATOXIN INDUCED SHOCK

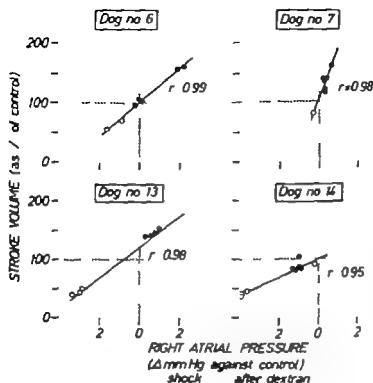
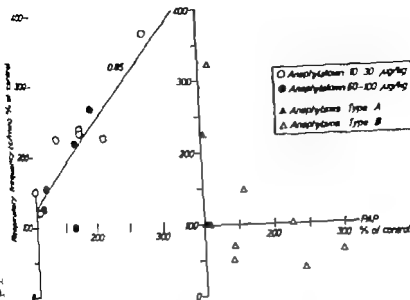


Fig. 5. Ventricular function curve after AT given in dose of 10, 25, 50, 30 $\mu\text{g/kg}$ in dogs no. 6, 7, 13, 14.

The first and second challenging injections were separate by 45 to 60 min. Similarly in dogs there was no response to the second injection of AT. In two dogs unresponsive to a second injection of horse serum, it was still possible to induce a shock by AT. However here the pulmonary hypertension was attenuated. Further in two dogs unresponsive to a second injection of AT it was still possible to induce a profound anaphylactic shock (type I). Thus tachyphylaxis against both horse serum and AT was observed but there was no cross tachyphylaxis between the anaphylactic agent and AT.

Discussion

The first important finding in this report is the occurrence of a shock state with cardiac output depressed to about 50 per cent after injections of AT in normal dogs and to about 25 per cent after injections of the anaphylactic agent in sensitized dogs. The decrease in cardiac output has obviously the same common cause in both AT induced and anaphylactic shocks: the primary portal venous blood pooling is followed by decreased venous return, decreased cardiac output and systemic arterial hypotension. This mechanism, which is typical for the dog, was described in ascaris-hypersensitive dogs already in 1951 by Graña *et al*. The role of the portal pooling seems to be essential since we could prove the shock response to the anaphylactic agent injected into the right atrium in sensitized dogs by a balloon occlusion of the inferior vena cava and thoracic aorta.



6. Respiratory frequency at the highest level of pulmonary artery pressure in 3 types of shock.

The second of our findings is that one set of cardiorespiratory patterns was regularly found in dogs sensitized over a short period with the anaphylactic agent only; somewhat different ones occurred in dogs sensitized over a longer period with the agent in Freund's serum; again another set of changes was associated with the AT-induced shock. Our findings also indicate that different mechanisms were involved in the anaphylactic and AT-induced shocks. This suggestion is supported by the demonstration of a complete hypophoria to the anaphylactic agent and (ii) AT in both isolated guinea pig lungs and in dogs—though under conditions of tachyphylaxis to one of the two agents, a full response could be sustained by the other agent. The presently observed effects of AT on the pulmonary and systemic circulations are similar to findings of Bodammer (1959) in the guinea pig and cat. Bodammer concluded that these effects of AT are direct & not mediated by released histamine or prostaglandins. In our experiments histamine & prostaglandins were released after both the anaphylactic agent and AT SRS, however released only in the anaphylaxis. Since AT readily released histamine and prostaglandins when injected into the pulmonary circulation, it may act both directly and via these mediators. The fact that indomethacin inhibited the AT-induced hypotension & tachypnea suggests that some product(s) of metabolism of arachidonic acid by cyclo-oxygenase was involved in this response. Further it appears that the titer of antibodies and the associated state of hypersensitivity are important determinants of the cardiorespiratory response in the anaphylactic shock. Dogs with high titers of hemagglutinating antibodies have a strong tendency towards a major and sustained elevation of the pulmonary artery pressure and decreased cardiac contractility; such changes were not observed in

ANAPHYLATOXIN INDUCED SHOCK

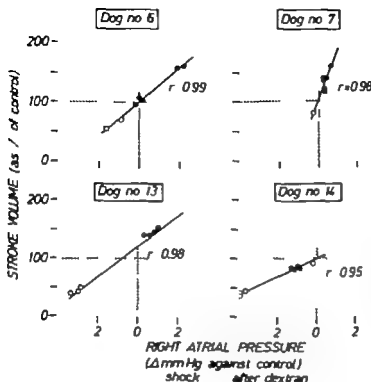


Fig. 5. Ventricular function one hour after AT given in dose of 10, 30, 50, 50 $\mu\text{g}/\text{kg}$ in dogs no. 6, 7, 13, 14.

The first and second challenging injections were separate by 45 to 60 min. Similarly in dogs there was no response to the second injection of AT. In two dogs unresponsive to the second injection of horse serum, it was still possible to induce a shock by AT. However here the pulmonary hypertension was attenuated. Further in two dogs unresponsive to the second injection of AT it was still possible to induce a profound anaphylactic shock (type II). Thus tachyphylaxis against both horse serum and AT was observed but there was no cross-over tachyphylaxis between the anaphylactic agent and AT.

Discussion

The first important finding in this report is the occurrence of a shock state with cardiac output depressed to about 50 per cent after injections of AT in normal dogs and to about 25 per cent after injections of the anaphylactic agent in sensitized dogs. The decrease in cardiac output has obviously the same common cause in both AT-induced and anaphylactic shocks: the primary portal venous blood pooling is followed by decreased venous return, decreased cardiac output and systemic arterial hypotension. This mechanism, which is typical for the dog, was described in ascaris-hypersensitive dogs already in 1947 by Grafia *et al*. The role of the portal pooling seems to be essential since we could prevent the shock response to the anaphylactic agent injected into the right atrium in sensitized dogs by a balloon occlusion of the inferior vena cava and thoracic aorta.

- and, M. J. SYMONS, and B. SAMUELSSON, Thromboxanes. A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. nat. Acad. Sci. (Wash.)* 1975. 72. 2994-8.
2. J. F. and R. P. TUNSON, Anaphylaxis. Course, mechanisms and treatment. *J. Amer. med. Assoc.* 227 1401-6.
3. J. M. D. DELLEBAND, G. SCHMIDT and W. VOOT. Structural elements of anaphylatoxins tested by contact activation of hog serum. *Happel-Seyler' Z. Physiol. Chem.* 1972. 353. 385-92.
4. R. P. and E. G. MOORE, The effect of thiols on the immunological release of slow reacting substance of anaphylaxis. II. Other *in vitro* and *in vivo* models. *J. Immunol.* 1976. 116. 392-6.
5. L. Studies of thermodilation technique for determination of cardiac output. *Acta Universitatis Faculty of Med.* 1974. 174.
6. K. Anaphylactic shock in the monkey: its hemodynamics and mediators. *Acta anaesth. scand.* 71 21 293-307.
7. P. J. Release and metabolism of prostaglandins in lung tissue. *Pol. J. Pharmacol. Pharm.* 1974. 26. 72.
8. P. J. and I. R. V. Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory substances. *Nature (Lond.)* 1969. 223. 29-35.
9. R. A. C. Anaphylatoxin-induced release of substances with prostaglandin-like activity in isolated blood vessels pig lungs. *Brit. J. Pharmacol.* 1972. 45. 544P-45P.
10. K. and B. URYLA, Purification and properties of the slow reacting substance formed in the *in vivo* perfused rat compound 42,80. *Acta physiol. scand.* 1971. 82. 358-74.
11. B. H. and S. RAVEN, Complement-dependent anaphylactic reactions. *Infection and Immunity* 1973. 1294-99.
12. L. The release and fate of vaso-active hormones in the circulation. *Brit. J. Pharmacol.* 1969. 35. 4-12.

dogs with low titers of antibodies. This may be related to several published findings. The anaphylactic process can be started via several immunological pathways (Ton and Raffel 1975) and the time period between immunization and challenge can also be important. For example a moderate type of degranulation of mast cells of mice occurs in the first 4 weeks whereas an explosive type of degranulation predominates during 5 to 8 weeks (Barnett and Justus 1975). Different mediators may be responsible for anaphylactic changes in different times from the start of sensitization. Flurbiprofen, an antagonist of SRS-A induced contraction of guinea pig tracheal chain protected guinea pigs against aerosol-induced anaphylactic shock during 3-4 weeks after sensitization but was ineffective during 5-8 weeks after sensitization (Greig and Griffin 1975). The sensitivity of the smooth muscle to mediators may vary considerably according to the immunological state. For instance toxicity of bradykinin is much higher in rats sensitized with horse serum or Bordetella pertussis vaccine than in controls. The vaccine itself had a weaker and more transient effect (Dawson and West 1965).

The depressed ventricular function observed during the shock only in dogs sensitized with horse serum and Freund's adjuvant can be explained partly by the loading effect of the pulmonary vasoconstriction, the major cause of the left-sided failure, however appears to be a direct affection of the heart by the anaphylactic process. One mediator deserves a consideration as a causative agent is SRS-A. This substance is released when antigen reacts with IgG_a antibodies (Orange and Moore 1976). It is biologically active in sub-nanogram quantities although its effects on the circulation have not been well documented. However when SRS from cat paw (Strandberg and Ulvén 1971) (which is very similar to SRS-A) was infused into the isolated perfused guinea pig heart it induced both coronary constriction and depression of contractility (Bernauer W. and Strandberg, K., personal communications about pilot experiments) and SRS-A may act similarly.

The kind help and advice of P. Borwell, H. Hedén and O. Tangen in connection with the preparation of anaphylatoxin is much appreciated.

References

- BARNETT, J. B. and E. E. JUSTUS, Differential effects of homocytotropic antibodies on the mast cell anaphylactic responses in actively and passively sensitized mice. *Infection and Immunity* (1975) 1342-51.
- BODANZIN, G. Untersuchungen über den Mechanismus der Blutdruckwirkung des Anaphylatoxins in Katzen und Meeresschnecken. *Neuro-Schneiderbergs Arch. Pharmac. exp. Path.* 1969, 262, 197-207.
- COLLIER, J. G., A. G. HERMAN and J. R. VANE, Appearance of prostaglandins in the renal vessels of dogs in response to acute systemic hypotension produced by bleeding or endotoxin. *J. Physiol. (Lond.)* 1973, 230, 19P-20P.
- DAWSON, W. and G. B. WEST, The importance of bradykinin in anaphylactic shock. *J. Pharm. Pharmacol.* 1965, 17, 246-47.
- FERRIERA, S. H. and J. R. VANE, The detection and estimation of bradykinin in the circulating blood. *Brit. J. Pharmacol.* 1967, 29, 367-77.
- GRAFA, A., F. C. MANN and H. E. ESSEX, Influence of the liver on the shock produced by extracerebral parasites. *Amer. J. Physiol.* 1947, 148, 243-52.
- GREIG, K. and B. SAMUELSSON, Prostaglandins and related factors. XIX. Thin-layer chromatography of prostaglandins. *J. Lipid Res.* 1964, 5, 117-20.
- GREIG, M. E. and R. L. GRIFFIN, Antagonism of slow reacting substance A anaphylaxis (SRS-A) and other spasmogens on the guinea pig tracheal chain by hydratropic acids and their effects on anaphylactic shock. *J. med. Chem.* 1975, 18, 112-116.

(for reviews see Ågren and Ronquist 1976). These involved either the endogenous protein kinase as an intrinsic acceptor or cases where both the enzyme and the acceptor were exogenous. Few of these protein kinase-mediated reactions have been cyclic AMP independent (Andrew *et al.* 1973 Bacalao and Rieber 1973 Ho *et al.* 1975 Steiner 1975, *et al.* 1975, Prasad *et al.* 1976). This includes the plasma membrane phosphorylation in for the Ehrlich, glioma and glioma cells (Ågren and Ronquist 1974 Ronquist and Ågren 1974a, 1974b, Ågren and Ronquist 1976) as well as 3T3 cells, both normal and 3-transformed (Mastro and Rozengurt 1976).

Many cyclic AMP-dependent protein kinases are known to be activated by more than one cyclic nucleotide. Cyclic GMP-specific kinases have in recent years been found in pig (Kao *et al.* 1976), guinea pig fetal lung (Nakazawa and Sano 1975), bovine adrenal (Shen *et al.* 1974), rat pancreas (Leemput *et al.* 1973) and cerebellum (Holman and Sold 1972), mammalian smooth muscle (Casnellie and Greengard 1974) and heart (Kao *et al.* 1972, 1976). Smooth muscle is the only tissue in which any endogenous acceptor proteins for cyclic GMP-dependent protein kinases have so far been found (Greenfield 1976).

The cyclic GMP-stimulated protein kinase differs from that stimulated by cyclic AMP in several of its physicochemical properties (Nakazawa and Sano 1975 Nishiyama *et al.* 1975, Takai *et al.* 1975). Nevertheless, enzymatic properties for both types of protein kinases are the same in respect to the requirements for the binding steps at the 3 different sites in the nucleotide, namely the binding of cyclic nucleotide for activation, the binding of ATP substrate and the binding of protein substrate for the reaction (Kobayashi and Greengard 1976).

The aim of the present study was to further characterize the endogenous protein kinase acceptor(s) of the plasma membrane vesicles obtained by the further purification by Dextran T 150 gradient centrifugation of a membrane preparation (Ronquist and Christensen 1973) from Ehrlich cells.

Materials and methods

Dextran T 150 was obtained by courtesy of Dr B. Lagergren, Pharmacia Fine Chemicals AB, Uppsala. Adenosine-5'-triphosphate (ATP) (sodium salt), dibutyryl cyclic AMP and GMP were purchased from Sigma Chemical Company St. Louis, Mo. γ -³²P-ATP was from NEN Chemicals, GmbH, Frankfurt-on-Main, W. Ger. The ingredients of the KRB buffer were products from E. Merck AG, Darmstadt, Germany. All reagents were of analytical grade. Human albumin (No. 58067) was from KABI AB, Stockholm, Sweden. *Preparation of tumour cells and plasma membrane fraction.* The Ehrlich ascites tumour cells were grown in 2-3 days in 5-week-old male Swiss albino mice obtained from the Anticancer Breeding Farm, Norrby, Stockholm. The tumour cells were separated by centrifugation of the ascites fluid, which had been allowed to stand 18-48 h for cold Krebs-Ringer bicarbonate (KRB) medium in order to diminish toxicity of cell agglutination. The cells were washed twice in the KRB-medium. Final washing was done in micro (0.25 M)-tris-glycyl-glycine (0.016 M) buffer pH 8.0, also containing 2 mM EDTA, followed by centrifugation. This was carried out in the International Refrigerated Centrifuge by the preparatory procedure of Ronquist and Christensen (1973) was followed exactly. A further purification of the membrane fraction was achieved by applying membrane material corresponding to about 45 mg protein in Dextran T 150 gradient. The gradient was composed by mixing equal parts of pure sodium phosphate-magnesium-buffer and the same buffer containing 19% w/v of Dextran

Phosphorylation of endogenous proteins by endogenous protein kinase of density gradient—purified plasma membrane vesicles of Ehrlich cells

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Abstract

RONQUIST G G ÅGREN and I LINDQVIST *Phosphorylation of endogenous proteins by endogenous protein kinase of density gradient—purified plasma membrane vesicles of Ehrlich cells* Acta physiol. scand. 1979 105 404-413

A phosphorylation of endogenous acceptor protein(s) has been demonstrated to occur in membrane vesicles prepared from Ehrlich ascites tumour cells. The reaction was catalyzed by endogenous protein kinase in the presence of exogenous (γ -³²P)ATP. A considerable increase of the specific protein kinase activity took place when the plasma membrane preparation was subjected to a further gradient centrifugation in De la Cruz T 150. This was done in the presence of a slightly alkaline phosphate buffer containing Mg-ions which resulted in the formation of a well defined vesicular preparation at density 1.03 g/ml gradient. The apparent K_m and V_{max} for the reaction with vesicles and exogenous (γ -³²P)ATP were determined and found to be 0.022 mM and 0.23 nmol $\text{mg}^{-1} \cdot 10 \text{ min}^{-1}$ respectively. Neither cyclic AMP nor cyclic GMP did stimulate the protein kinase-catalyzed reaction. Instead, a clear inhibition of the reaction by the cyclic nucleotides was unexpectedly registered. Adenosine at 0.5 mM also inhibited the reaction. Calcium ions were inhibitory at all concentrations tested in the presence of a fixed (γ -³²P)ATP/Mg²⁺ ratio. When Mg-ions were stoichiometrically replaced by Ca-ions practically no activity was observed.

The presence of endogenous protein kinase at the surface of Ehrlich cells catalyzing the phosphorylation of endogenous membrane protein(s) was first described (1970 (Ågren and Ronquist 1970; Ronquist and Ågren 1970)) and other reports followed (Ågren and Ronquist 1971; Ronquist and Ågren 1974 a, 1974 b; Ågren and Ronquist 1976; Ronquist et al. 1977). Schlatz and Marinetti (1971) described the cyclic AMP dependent phosphorylation of isolated rat liver plasma membrane by a partially purified protein kinase from rat liver. In a similar experiment Kinzel and Mueller (1973) were able to demonstrate the phosphorylation of endogenous acceptor proteins at the surface of intact HeLa cells in the presence of a protein kinase isolated from rat skeletal muscle and (γ -³²P)ATP in the external medium. This reaction was also stimulated to a limited degree by cyclic AMP. Protein kinase-catalyzed membrane phosphorylations have subsequently been published for many membrane systems.

11.1 Protein content of Ehrlich cells and membrane subfractions. 3.6×10^8 Ehrlich cells were used for the preparation of plasma membrane fraction. Subsequent purification followed by gradient centrifugation of the plasma membrane fraction resulting in the recovery of membrane vesicles at density 1.03–1.04 of the gradient.

µg	Protein content, in mg	Per cent of total
lysate of Ehrlich cells (3×10^8)	1 053	100
plasma membrane fraction	44.8	4.3
membrane vesicles after gradient centrifugation	2.0	0.19

Results

Fig. 1 illustrates the protein content of a homogenate from intact Ehrlich cells. During course of the work it turned out to be most convenient to start with 3.6×10^8 Ehrlich cells corresponding to about 1 050 mg protein (1 ml of packed Ehrlich cells is equivalent to 1.5×10^8 cells). The protein content of the plasma membrane preparations before gradient centrifugation was 4.3 % of that for the Ehrlich cells corresponding to about 23 times purification. The protein content of the vesicular material of the main component after gradient centrifugation implied another 23 times purification. The vesicular nature of the material separated from the main component at density 1.035 in the gradient (Fig. 1) is clearly seen in Fig. 2. The vesicles vary in size (Fig. 2 a) and are bounded by a typical three-layered membrane, 7–9 nm thick (Fig. 2 b).

Table II shows a considerable increase in specific activity for membrane vesicles, i.e. (^{32}P) phosphoryl group transfer into phosphorylserine- and phosphorylthreonine-residues of membrane protein. The (^{32}P) phosphoryl group transfer reaction from exogenous $\gamma\text{-ATP}$ into endogenous acceptor(s) of the membrane vesicles is a relatively slow process seen in Fig. 3, and an incubation time of 10 min still represents initial velocity under the conditions used. A Lineweaver and Burk graph for the phosphoryl group transfer reaction seen in Fig. 4. The apparent K_m is 0.022 mM and the apparent V_{max} is $0.23 \text{ nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$. 0.5 mM adenosine inhibits the transfer reaction by about 45 % and this inhibition is not further increased by a higher adenosine concentration (Table III).

11.2 Protein kinase activity in plasma membrane fraction and membrane vesicles obtained by gradient centrifugation. Experiments performed in basic incubation medium containing 0.05 mM $\gamma\text{-ATP}$ and 4 mM Mg^{2+} for 10 min at 37°C. Values are given in pmol (^{32}P) phosphorylserine- and (^{32}P) phosphorylthreonine-residues recovered per mg of protein.

µg	(^{32}P) phosphoryl- serine, pmol/mg	(^{32}P) phosphoryl- threonine, pmol/mg
plasma membrane fraction	1.1	0.23
membrane vesicles obtained by gradient centrifugation	164	34.9



Fig. 1 Separation of plasma membrane material upon density gradient centrifugation resulting in the recovery of a main component consisting of 3 scanty layers at density 1.03–1.04. For details, see text.

T 150. The strength of the buffer was $5 \cdot 10^{-4}$ M as regards sodium phosphate and $1 \cdot 10^{-4}$ M as regards magnesium sulfate. The pH of the buffer was 8.0. Centrifugation proceeded for 18 h at 23 000 rpm in MSE Super speed 65 centrifuge Rotor SW 3 25 (60 000 g). A main membranous component, consisting of 3 scanty layers was recovered at a density-interval of 1.03–1.04 of the gradient (Fig. 1). Three layers of the main component were carefully removed and suspended in 130 mM NaCl and 25 mM KCl and centrifuged for 45 min at 30 000 rpm in the MSE Super speed 65 centrifuge. The pellet which was resuspended in 8 ml of 130 mM NaCl and 25 mM KCl before incubation.

Incubation procedure. The basic incubation medium was the following in a final volume of 10 ml containing the isotonic vesicular suspension of 130 mM NaCl and 25 mM KCl, 350 μ mol Tris-acetate buffer pH 7.5, 5 μ mol glutathione (reduced form), 40 μ mol $MgCl_2$, 10 μ mol of unlabeled orthophosphate (disodium form) usually 250–500 nmol of (γ - ^{32}P)ATP (Spec. act. 0.5 μ Ci/nmol). The vesicular material corresponded to 2–4 mg protein.

The membrane vesicles were prewarmed for 4 min at 37° in an atmosphere of 93.5% oxygen and 6.5% carbon dioxide before incubation. In the experiments where Mg -ions were replaced by Ca -ions, the latter were included during the 4 min prewarming step. Incubation proceeded for various times, generally 5–10 min, and was terminated by the addition of 10 ml of 20% trichloroacetic acid (TCA). Just before TCA precipitation 50 mg of human albumin were added to the incubation medium to co-precipitate the small amounts of membrane material. To achieve a complete precipitation the deaerated material kept for at least 16 h at 4°C in the TCA-solution before centrifugation. The pellet was washed with 10% TCA containing an excess of unlabeled ATP and orthophosphate.

Analytical methods. The washed precipitate was dried in ethanol and then followed by partial hydrolysis according to Lipmann (1933), with 10 times the protein weight of 2 M HCl under reflux on a water bath for 20 h. The HCl was removed by repeated evaporations (4 times) *in vacuo* and the hydrolysate was supplied with 1 mg of unlabeled phosphorylserine, 1.5 mg of phosphorylthreonine and 1.5 mg of orthophosphate and applied on a 50 ml Dowex 50 column. Elution was carried out with 0.01 M HCl and labeled phosphorylserine- and phosphorylthreonine-residues were isolated and identified (Ågren and Reynolds 1970).

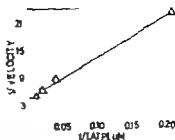
Radioactivity was determined in a Nuclear Chicago Scintillation Counter and (^{32}P)radioactivity determined by measuring the Cerenkov radiation.

Protein was determined according to Lowry *et al.* (1951) with bovine serum albumin as standard.

Electron microscopy. The main component at density 1.035 in the gradient usually consisting of 3 layers was collected by withdrawing 2 ml fractions. Each fraction was resuspended in excess of the sodium phosphate-magnesium-buffer pH 8.0, and washed twice. Each washing was performed by centrifugation in a Spinco L50 centrifuge rotor 40 f r 10 min at 20 000 rpm. The washing procedure was followed by fixation in glutaraldehyde (2.5% glutaraldehyde in the sodium phosphate-magnesium-buffer) for 20 min at room temperature. Washing twice followed in the sodium phosphate-magnesium-buffer and centrifugation in the Spinco centrifuge for 10 min at 20 000 rpm. The material was then fixed in OsO₄ (final concentration was 1% in the above-mentioned buffer) for 20 min. Two washings followed in this buffer.

Dehydration was started with 50% ethanol solution and performed with increasing concentrations of ethanol up to 100% and followed by propylene-oxide treatment before embedding in Epon (Laird 1968). During the course of fixation and dehydration, the membranous material was transformed into a dense localization, and centrifugation at low speed was necessary at each step of the procedure, because of its very slow sedimentation by gravity alone. Sections were cut stained by uranyl acetate and lead citrate (Reynolds 1963) and examined in Philips EM 301.

Fig. 1 A Lineweaver-Burk graph of the protein kinase activity assayed in the recovery of (32 P)phosphorylserine residues, of vesicles obtained by gradient centrifugation. The (γ - 32 P)ATP/total was maintained constant. Incubation proceeded for 10 min at 37°C.



Discussion

4.4 per cent of the total cell protein content was recovered in the plasma membrane fraction. The subsequent gradient centrifugation in Dextran T 130 resulted in a further fraction implying again a 4.4 per cent recovery calculated on a protein basis. The specific kinase activity of the vesicular membrane material, the main component was variable and exceeded that of the plasma membrane fraction by more than 140 times. Two explanations might exist. One is the homogenous vesiculation of the membrane and on gradient centrifugation in the slightly alkaline Mg^{2+} -containing buffer. It means the prerequisite conditions were available for the same enzymes for all vesicles. Further, it is reasonable to assume that the protein kinase is at the outer surface of the vesicles and that the vesicles are sealed and impermeable for (γ - 32 P)ATP (cf Glynn 1968, and Rönquist 1969). We have previously demonstrated the presence of endogenous

Fig. 2 Effect of two different concentrations of adenosine on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.025 mM (γ - 32 P)ATP and 2 mM Mg^{2+} together with 0.5 mM and 1 mM adenosine, respectively for 5 min at 37°C. Activity is expressed in pmol (32 P)phosphorylserine- and (32 P)phosphorylthreonine-residues recovered per mg protein.

Adenosine No.	(32 P)phosphorylserine, pmol/mg	(32 P)phosphorylthreonine, pmol/mg
Control, no adenosine added	109	28.3
1. plus 0.5 mM adenosine	61.4	16.0
2. plus 1 mM adenosine	64.6	9.1

Fig. 3 Effect of dibutyryl cyclic AMP and dibutyryl cyclic GMP on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.025 mM (γ - 32 P)ATP and 2 mM Mg^{2+} together with $1 \cdot 10^{-6}$ M dibutyryl cyclic AMP or $1 \cdot 10^{-6}$ M dibutyryl cyclic GMP for 5 min at 37°C. Activity is expressed in pmol (32 P)phosphorylserine- and (32 P)phosphorylthreonine-residues recovered per mg protein.

Adenosine No.	(32 P)phosphorylserine, pmol/mg	(32 P)phosphorylthreonine, pmol/mg
Control, no cyclic nucleotide added	106.3	35.3
1. plus $1 \cdot 10^{-6}$ M cAMP	61.4	21.8
2. plus $1 \cdot 10^{-6}$ M cGMP	33.9	23.6

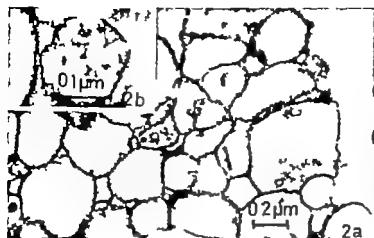


Fig. 2. Electron micrographs of membrane material recovered at density 1.035 of the Dextran T15 gradient. Vesicles dominate (Fig. 2a) and are bounded by a 7-9 μ thick membrane (Fig. 2b, see Magnifications. 2a \times 50 000, 2b \times 200 000).

In accordance with previous findings on intact Ehrlich cells and a plasma membrane preparation from these cells (Ronquist and Ågren 1974a, Ågren and Ronquist 1976, Ronquist *et al.* 1977) cyclic AMP and cyclic GMP did not stimulate the protein kinase reaction in the membrane vesicles (Table IV).

Instead an unambiguous inhibition by about 40-50 per cent was achieved by the cyclic nucleotides. Table V illustrates the influence of Ca^{2+} ions on the incubation system at different concentrations. Keeping the $(\gamma\text{-}^3\text{P})\text{ATP}/\text{Mg}^{2+}$ concentrations constant at 0.025 mM and mM respectively increasing Ca^{2+} concentrations resulted in enhanced inhibition of the protein kinase reaction. At 5 mM Ca^{2+} concentration the inhibition amounted to about 10 per cent and when all Mg^{2+} were stoichiometrically replaced by Ca^{2+} the inhibition was nearly 100% (Table V). Therefore the membrane-linked protein kinase had an absolute requirement of Mg^{2+} vis-à-vis Ca^{2+} .

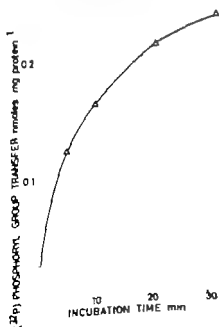


Fig. 3. Time course of protein kinase reaction, as measured by the recovery of (^3P) phosphorylcholine residues of the vesicles obtained by gradient centrifugation. Experiments performed in basic incubation medium containing 0.05 M $(\gamma\text{-}^3\text{P})\text{ATP}$ and 4 mM Mg^{2+} at 37°C .

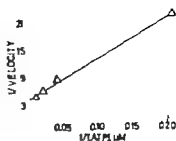


Fig. 1. A Lineweaver-Burk graph of the protein kinase activity assayed as the recovery of (^{32}P) phosphorylserine residues, of which obtained by gradient centrifugation. The $(\gamma\text{-}^{32}\text{P})\text{ATP}$ /assay was maintained constant. Incubation proceeded for 5 min at 37°C .

Discussion

44 per cent of the total cell protein content was recovered in the plasma membrane fraction. The subsequent gradient centrifugation in Dextran T 150 resulted in a further fraction implying again a 4.4 per cent recovery calculated on a protein basis. The specific kinase activity of the vesicular membrane material of the main component was notable and exceeded that of the plasma membrane fraction by more than 140 times. Two explanations might exist. One is the homogeneous vesiculation of the membrane material on gradient centrifugation in the slightly alkaline Mg^{2+} -containing buffer. It means that the prerequisite conditions were available for the same enzymes for all vesicles. Further, it is reasonable to assume that the protein kinase is at the outer surface of the vesicles and that the vesicles are sealed and impermeable for $(\gamma\text{-}^{32}\text{P})\text{ATP}$ (cf Glynn 1968, and Rosquist 1969). We have previously demonstrated the presence of endogenous

Table I. Effect of two different concentrations of adenosine on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.05 mM $(\gamma\text{-}^{32}\text{P})\text{ATP}$ and 4 mM Mg^{2+} together with 0.5 mM and 1 mM adenosine, respectively. For 5 min at 37°C . Activity is expressed in pmol (^{32}P) phosphorylserine- and (^{32}P) phosphorylthreonine-residues recovered per mg protein.

Incubation No.	(^{32}P) phosphorylserine, pmol/mg	(^{32}P) phosphorylthreonine, pmol/mg
Control, no adenosine added	109	28.3
1 plus 0.5 mM adenosine	61.4	16.0
2 plus 1.0 mM adenosine	64.6	9.1

Table II. Effect of dibutyryl cyclic AMP and dibutyryl cyclic GMP on the protein kinase of vesicles purified by gradient centrifugation. The experiments were performed in the basic incubation medium containing 0.025 mM $(\gamma\text{-}^{32}\text{P})\text{ATP}$ and 2 mM Mg^{2+} together with $1 \cdot 10^{-4}$ M dibutyryl cyclic AMP or $1 \cdot 10^{-4}$ M dibutyryl cyclic GMP for 5 min at 37°C . Activity is expressed in pmol (^{32}P) phosphorylserine- and (^{32}P) phosphorylthreonine-residues recovered per mg protein.

Incubation No.	(^{32}P) phosphorylserine, pmol/mg	(^{32}P) phosphorylthreonine, pmol/mg
Control, no cyclic nucleotide added	106.3	35.3
1 plus $1 \cdot 10^{-4}$ M cAMP	61.4	21.8
2 plus $1 \cdot 10^{-4}$ M cGMP	53.9	29.6

TABLE V Effect of different concentrations of Ca^{2+} on the protein kinase of vesicles purified by pulse centrifugation. The experiments were performed in the basic incubation medium containing 0.025 mM (γ - ^{32}P)ATP and 2 mM Mg^{2+} except in experiment No. 6 where Mg^{2+} was stoichiometrically replaced by Ca^{2+} together with 4 different concentrations of Ca^{2+} for 5 min at 37°C. Activity is expressed in pmol (^{32}P)phosphorylserine- and (^{32}P)phosphorylthreonine-residues covered per mg protein.

Experiment No.	(^{32}P)phosphorylserine pmol/mg	(^{32}P)phosphorylthreonine, pmol/mg
1. Control, no Ca^{2+} added	110.3	23.7
2. As 1 plus 0.5 mM Ca^{2+}	114.6	22.7
3. As 1 plus 1.5 mM Ca^{2+}	70.2	14.9
4. As 1 plus 3.0 mM Ca^{2+}	34.3	7.8
5. As 1 plus 5.0 mM Ca^{2+}	23.8	3.3
6. As 1., but all Mg^{2+} replaced by Ca^{2+}	1.23	0.21

The membrane vesicles were pre-incubated for 4 min with the Ca -ions (2 mM).

protein kinase at the outer surface of intact Ehrlich cells as well as of glia and plasma (Ronquist and Ågren 1974 a, 1974 b, Ågren and Ronquist 1976, Ronquist *et al.* 1976, Ågren and Ronquist 1978) and this observation has been confirmed by others on other types (Mastro and Rozengurt 1976, Schlaeger and Köhler 1976). This finding is in accordance with the fact that the vesiculation was performed under conditions that favoured right side-out formation in human erythrocytes (Kant and Steck 1972) are in accordance with the view that the vesicles studied are most probably right side-out contrary to the vesicles from the plasma membrane preparation, that might be inside-out (Wernstedt *et al.* 1975).

Determination of possible adenyl cyclase as a marker enzyme was carried out according to Wikström and Ågren (1973). A slight adenyl cyclase activity was registered with vesicles. This activity was increased 4 times when including Triton in the incubation medium favouring the view that the vesicles were right side out. Thus, Triton facilitated the entry of the ATP to the catalytic site of the membrane-bound enzyme system.

The other explanation could be the dissociation of a possible inhibitor to the protein kinase on gradient centrifugation or revealing of an active site of the enzyme being "cryptic" prior to this treatment. Also the stoichiometric relationship between the protein kinase and phosphoprotein-phosphatase molecules (*cf.* Ågren and Ronquist 1978) present at the surfaces of the intact cells and the vesicles might have changed so that proportionally more protein kinase molecules than phosphoprotein phosphatase molecules are present at the surface of the vesicles. If such were the case, it also explains the time extension for maximal phosphorylation of the vesicles compared with intact cells (Ågren and Ronquist 1978). In accordance with previous work cyclic nucleotides failed to stimulate the protein kinase activity of the vesicles (Ronquist and Ågren 1974 a, 1974 b, Ågren and Ronquist 1976, Mastro and Rozengurt 1976, Ronquist *et al.* 1977). Instead, a clear inhibition by both dibutyryl cyclic AMP and dibutyryl cyclic GMP was observed.

The aliphatic dibutyryl analogues of the cyclic nucleotides were used since they can penetrate the membranous structure. This was appropriate, since a possible effect by cyclic nucleotides could be achieved regardless of a *cis*- or *trans*-position of the NH_2 group.

decide is δ vs the substrate ATP. The inhibitory effect by the dibutyryl derivatives did not be explained simply by a chelating reaction with Mg^{2+} since this divalent cation is present in a great excess. Furthermore, one might argue that the cyclic nucleotides could be hydrolyzed since no diesterase inhibitor was present in the incubation system. However, an approximate 50% reduction of the cellular content of the diesterase could be ascribed to the highly purified vesicles (*cf* ref Cheung 1967). Also, no inhibitory effect was to be anticipated when most of the cyclic nucleotides were hydrolyzed, since 5 AMP at 10^{-4} M concentration in the incubation medium did not influence the reaction (unpublished observation). This finding was unexpected but in accordance with recent reports Gern and Kang (1977) as well as by Lucid and Griffin (1977) who studied other plasma membrane fractions and found that cyclic AMP clearly decreased the cyclic AMP independent phosphorylation of membrane proteins in their experimental systems.

Also, the inhibitory effect by 0.5 mM adenosine was congruous to the results from an intracellular system of beef thyroid tissue involving a cyclic AMP dependent protein kinase (Simpson and Field 1976).

The findings that incorporation of the (γ - ^{32}P)phosphoryl group of ATP into phosphorylated and phosphoryl threonine is inhibited to different extent by adenosine, the dibutyryl nucleotides and Ca^{2+} suggest that more than one protein kinase is present in the membrane.

A cyclic nucleotide dependency of the protein kinase at the outer surface of the membrane does not appear to be obligatory for protein kinase being involved in a regulatory phosphorylation function. Instead, we propose an autoregulatory mechanism based upon the Ca^{2+}/Mg^{2+} ratio in the microenvironment of the membrane. An additional component of the autoregulatory mechanism might be the possible phosphoprotein phosphatase activity which is higher in the presence of Ca^{2+} than with Mg^{2+} contrary to the protein kinase activity. There is evidence that the phosphorylation of the surface membrane occurs also *in vivo* (Ågren and Ronquist 1978) and a transfer of phosphoryl groups can take place between cells (Ågren and Ronquist 1978). It has been claimed that exogenous ATP can influence the permeability properties of some cells (Hempling *et al* 1969) and such an influence might be mediated by a phosphorylation of the surface membrane (Garbit *et al* 1976, Simpson *et al* 1977).

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References

- Ågren, G. and G. Ronquist. Fermentation of extracellular adenosine triphosphate by tumor cells. *Acta physiol. scand.* 1969 75 124-129.
- Ågren, G. and G. Ronquist. Isolation of ^{32}P -labelled phosphorylserine from Ehrlich mouse-ascited tumor cells suspended in an isotonic medium containing ^{32}P -labelled adenosine triphosphate. *Acta physiol. scand.* 1970 79 125-128.
- Ågren, G. and G. Ronquist. Isolation of ^{32}P -labelled phosphorylserine and phosphorylthreonine from Ehrlich mouse-ascites tumor cells, suspended in an isotonic medium containing ^{32}P -labelled nucleotides or inorganic pyrophosphate. *Acta chim. scand.* 1971 25 2931-2934.

- ÅGREN, G and G RONQUIST (³²P)Phosphoryl transfer by endogenous protein kinase at the glia and fibroblast cell surface in culture into extrinsic acceptor proteins. *Acta physiol. scand.* 1974. 92. 436-437.
- ÅGREN, G and G RONQUIST Phosphorylation of endogenous membrane proteins by endogenous protein kinase at the outer surface of Ehrlich cells. *Uppsala J Med. Sci.* 1976. 81. 129-134.
- ÅGREN, G and G RONQUIST Phosphoryl group transfer from Ehrlich cells prelabelled with ³²P-orthophosphate and (¹⁴C)glucose to unlabelled glioma cells. *Acta physiol. scand.* 1972. 93. 129-130.
- ANDREW C., A. DI ROSSI, R. R. ALMON and S. APPEL, Phosphorylation of muscle membranes: identification of membrane-bound protein kinase. *Science* 1973. 182. 927-929.
- BACALO, J and M. REIER, On the properties of a membrane-associated protein kinase from Chinese hamster ovary cells. *FEBS Lett* 1973. 37. 37-41.
- CASSELL, J. E. and P. GREENGARD, Guanosine 3',5'-monophosphate-dependent phosphorylation of endogenous substrate proteins in membranes of mammalian smooth muscle. *Proc. nat. Acad. Sci. (Wash.)* 1974. 71. 1891-1895.
- CHUNG, W. Y., Properties of cyclic 3',5'-nucleotide phosphodiesterase from rat brain. *Biochem.* 1967. 6. 1079-1085.
- GATES, R. E. and E. S. KANG, cAMP Mediated decrease in membrane phosphorylation. *Biochem. biophys. Res. Commun.* 1977. 77. 606-610.
- GAZITT, Y., J. OHAD and A. LOTTY, Phosphorylation and dephosphorylation of membrane proteins: a possible mechanism for structural rearrangement of membrane components. *Biochem. biophys. Acta (Amst.)* 1976. 436. 1-14.
- GLYNN, L. M. Membrane adenosine triphosphatase and cation transport. *Brit. med. Bull.* 1968. 24. 163-168.
- GREENGARD, P. Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. *Nature (Lond.)* 1976. 260. 101-108.
- HEMPLING, H. G. C. C. STEWART and G. GASE, The effect of exogenous ATP on the electrolytic current of TA₃ sarcoma tumor cells. *J. Cell. Physiol.* 1969. 73. 133-140.
- HO, R., T. RUSSEL, T. ASAKURA and A. P. SNYDER, Influence on adipocyte plasma membrane bound protein kinase by feedback regulator J. *Cyclic Nucleotide Res.* 1975. 1. 349-358.
- HOFFMAN, F. and G. SOLD, A protein kinase activity from rat cerebellum stimulated by guanosine-3',5'-monophosphate. *Biochem. biophys. Res. Commun.* 1972. 49. 1100-1107.
- KANT, J. A. and T. L. STICK, Cation-impermeable inside-out or right-side-out vesicles from human erythrocyte membranes. *Nature New Biol.* 1972. 240. 26-28.
- KARIYA, T. and J. B. FIELD, Effects of adenosine and its derivatives on protein kinase activity of thyroid. *Biochem. biophys. Acta (Amst.)* 1976. 431. 41-47.
- KNEDEL, V. and C. G. MUELLER, Phosphorylation of surface proteins of HeLa cells using endogenous protein kinase and (³²P)ATP. *Biochem. biophys. Acta (Amst.)* 1973. 322. 337-351.
- KOBAYASHI, R. and V. FANO, Studies on cyclic GMP-dependent protein kinase properties by blue dextran sepharose chromatography. *Biochem. biophys. Res. Commun.* 1976. 69. 1080-1087.
- KUO, J. F. C. W. D. VU and J. TIA, Depressed cardiac cyclic GMP-dependent protein kinase in spontaneously hypertensive rats and its further depression by guanethidine. *Nature (Lond.)* 1976. 261. 37-39.
- KUO, J. F. W. N. KUO, M. SHOH, C. W. DAVES, V. L. SEERY and T. E. DONNELLY, Purification and general properties of guanosine 3',5'-monophosphate-dependent protein kinase from guinea pig adrenal. *J. Biol. Chem.* 1976. 251. 1759-1766.
- KUO, J. F. T. P. LEE, H. L. REYER, K. G. WALTON, T. E. DONNELLY and P. GREENGARD, Cyclic nucleotide dependent protein kinases X. An assay method for the measurements of guanosine 3',5'-monophosphate in various biological materials and study of agents regulating its levels in heart and brain. *J. Biol. Chem.* 1972. 247. 16-22.
- LEMPERT, M. J. CANUS and J. CHRISTOWSKI, Cyclic nucleotide-dependent protein kinase of the rat pancreas. *Biochem. biophys. Res. Commun.* 1973. 54. 182-190.
- LEPMANN, F. Über die Bildung der Phosphorsäure in Phosphoproteinen. I. Mitteilung: Isolierung der Phosphorhaltigen Aminosäure (Serinphosphorsäure) aus Casein. *Biochem. Z.* 1933. 262. 3-13.
- LOWRY, D. H. N. J. ROSEBROUGH, A. L. FAR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951. 193. 265-275.
- LUCIN, S. W. and M. J. GRIFFIN, Cyclic AMP inhibition of protein kinase activity in HeLa cell plasma membranes. *Biochem. biophys. Res. Commun.* 1977. 79. 1293-1298.
- LUFT, J. H., Improvements in epoxy resin embedding method. *J. Biophys. Biochem. Cytol.* 1961. 17. 404-411.

1. A. M. and E. ROSENBLUTH, Endogenous protein kinase in outer plasma membrane of cultured cells. *J. Biol. Chem.* 1974. 251: 7898-7909.
2. K. and M. SANO, Partial purification and properties of guanosine 3',5'-monophosphate-activated protein kinase from pig lung. *J. Biol. Chem.* 1975. 250: 7415-7419.
3. K., H. KATAKAMI, H. YAMAMURA, Y. TAKAI, R. SUMIMURA and Y. NISHIZUKA, Functional activity of guanosine 3',5'-monophosphate-dependent and adenosine 3',5'-monophosphate-dependent protein kinases from various sources. *J. Biol. Chem.* 1975. 250: 1297-1300.
4. K. N., M. POOLMAN, M. GASCHELE, P. K. SINHA and L. BROWN, Cyclic nucleotide-dependent protein kinase activity in malignant and cyclic AMP-induced "differentiated" neuroblastoma cells in culture. *Biochem. Biophys. Res. Commun.* 1976. 62: 1248-1254.
5. E. S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* 1961. 17: 208-212.
6. G. and G. AOKI, Isolation of 32 P-labelled phosphorylserine from Ehrlich tumor vesicles and cells suspended in an isotonic medium containing 32 P-labelled adenosine triphosphate. *Acta biochem.* 1970. 24: 722-729.
7. G. and G. AOKI, Isolation of 32 P-labelled phosphorylserine and phosphorylthreonine from Ehrlich tumor vesicles and cells suspended in different isotonic media containing 32 P-labelled adenosine triphosphate. *Acta biochem.* 1974. 28: 1169-1174.
8. G. and G. AOKI, 32 P-phosphoryl transfer by endogenous protein kinase at the Ehrlich cell vesicles and cytosolic acceptor proteins. *Uppsala J. Med. Sci.* 1974. 79: 158-162.
9. G. and H. N. CHATTERJEE, Adenosine acid phosphorylation of alkaline phosphatase-dependent ATP cleavage in Ehrlich cell membrane preparation. *Biochim. Biophys. Acta (Amst.)* 1973. 323: 337-341.
10. G. II AOKI, S. ELLIOTT and C. O. WERNER, Cyclic 3',5'-GMP-dependent protein kinase at the outer surface of intact Ehrlich cells. *Uppsala J. Med. Sci.* 1977. 82: 1-5.
11. E. L. A. HOFFEL and J. FARRAR, Effect of exogenous ATP on the permeability of transient release of mouse cell lines. *J. Biol. Chem.* 1977. 252: 4584-4590.
12. E. J. and G. KÖHLER, Essential cyclic AMP-dependent protein kinase activity in rat C-6 glioma. *Ph. Assay (Lond.)* 1976. 260: 705-707.
13. L. and G. U. MARINETTI, Protein kinase mediated phosphorylation of the rat liver plasma membrane. *Biochim. Biophys. Res. Commun.* 1977. 65: 51-56.
14. S. M. MITSUNAGA, Y. KAWAMURA, S. TACHIBANA and T. MARAO, Studies on cyclic nucleotides in the adrenal gland. III. Properties of cyclic AMP and GMP-dependent protein kinases in the adrenal gland. *Biochim. Biophys. Acta (Amst.)* 1976. 241: 56-64.
15. M. Endogenous phosphorylation of platelet membrane proteins. *Arch. Biochem.* 1975. 171: 205-204.
16. E. K. NISHIZUKA, H. YAMAMURA and Y. NISHIZUKA, Guanosine 3',5'-monophosphate-dependent protein kinase from bovine cerebellum. Purification and characterization. *J. Biol. Chem.* 1975. 250: 7419-7425.
17. M., N. VIGNAUD and P. MANDIL, Light-stimulated phosphorylation of rhodopsin in the retina. The presence of protein kinase that is specific for photobleached rhodopsin. *Proc. nat. Acad. Sci. (U.S.A.)* 1975. 72: 331-335.
18. C. O. G. AOKI and G. ROSENBLUTH, Enzyme activities at the surface of intact Ehrlich tumor cells with alterations in the isotonic assay medium. *Cancer Res.* 1975. 35: 1534-1541.
19. S. E. and G. AOKI, Some properties of adenosyl cyclase from Ehrlich cells. *Cancer Res.* 1977. 37: 2714-2722.

Progesterone effects on the evolution of myometrial activity in vivo in the non pregnant rabbit

By

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Abstract

BATRA, S., M. ÅKERLUND and T. LAUDANSKI. Progesterone effects on the evolution of myometrial activity in vivo in the non-pregnant rabbit. *Acta physiol. scand.* 1979 105: 414-419.

In order to study the influence of progesterone on the myometrial activity ovarian secretion of progesterone was stimulated by intravenous injection of human chorion gonadotrophin (HCG, 75 IU) in 9 nonpregnant rabbits. 4 rabbits, used as controls, were given only saline. Plasma progesterone concentration in these samples taken at frequent intervals (2-4 h) during the recording was measured by radioimmunoassay. In the controls, the myometrial activity and the plasma levels of progesterone did not change. After 1 IU HCG administration the amplitude of contraction began to decrease and the frequency began to increase after 3 h. After about 5 h the changes in amplitude and frequency were generally fully developed. The pattern of myometrial activity persisted throughout the recording period (12 h) and, as also proved by recordings made after 24 and 48 h of HCG injection. A maximal increase in plasma progesterone concentration (5.0 ng/ml) had already occurred by 2 h after the HCG injection and gradually decreased thereafter. It is concluded that an elevation in the level of progesterone in non-pregnant rabbits resulting from increased ovarian output (through HCG) has a pronounced and immediate depressing effect on the character of myometrial activity which is indicative of a shift from nonpregnant to pregnant type.

It is well known that the pattern of myometrial activity differs between the non-pregnant and pregnant conditions in animals as well as in humans. Generally in early pregnancy the uterine activity is weaker and more uncoordinated than in non-pregnant condition. Moreover in early pregnancy the uterus is relatively sensitive to stimulants (Laudanski *et al.* 1977). Although it has been widely speculated that progesterone is a major factor in bringing about the change in the pattern of myometrial activity from the non-pregnant to the pregnant type, there is little direct evidence showing that such an effect is in fact caused by exposure to increased concentration of progesterone (see Porter 1974). Furthermore it was suggested that there is a latency period in the action of progesterone even after an intravenous administration of 17-24 h (Schofield 1955; Caspo and Lloyd-Jacob 1961). The dose of progesterone generally used in these earlier studies was very large and far from physiological.

has been clearly demonstrated that an exogenous administration of human chorion gonadotropin (HCG) to rabbits causes marked and rapid changes in the ovarian steroid secretion, particularly of progesterone (Hilliard 1973, Mills *et al.* 1972). We chose this approach in the present study to investigate the progesterone induced changes in the myometrial activity. Within 1 h after an i.v. injection of HCG the levels of plasma progesterone, as well as of 20 α -dihydro-progesterone increase markedly (Hilliard 1973, Mills *et al.* 1972) and ovarian secretion of these steroids remains high for about 4-6 h and then declines to low levels after about 8 h, while approaching the ovulation time. An identical pattern of ovarian steroid secretion is observed after coltus-induced ovulation. By injecting HCG during a continuous recording of the myometrial activity in rabbits while also collecting blood samples for assay of progesterone before and at frequent intervals after the HCG injection we were able to demonstrate that an elevation in the plasma progesterone level was responsible for a change in the character of myometrial activity.

Methods

Twelve of the Swedish Land race weighing about 3 kg were used. They were non-pregnant but had all been mated at an earlier date. The rabbits were housed under standardized conditions of light and temperature and were fed freely with mixed diet and tap water. The myometrial activity was recorded as changes in intrauterine pressure. The pressure sensors were placed at laparotomy during anaesthesia with pentobarbitone sodium (Nobutoxalstron 6%, ACO, Sweden). In 9 of the does referred to as "acute" rabbits, the intrauterine pressure recordings were performed directly following the operation (Laudanski *et al.* 1977) by a micro-transducer catheter (Åkerlund & 1975). In another 3, referred to as "chronic" rabbits, the recordings were obtained at least one month after the placement of the pressure sensors. A recording system with sponge-tipped saline-filled polyethylene catheters (Laudanski 1966) at the tip in the middle of one uterine horn was used in this group. The intrauterine pressure recordings were analysed for frequency (number of pressure cycles during 1 min) and amplitude of the contractions. These calculations were done on 20-40 mm long parts of the recording at various intervals. The analysis was done by the same person and only on parts of the recording which showed no significant artefacts (resulting from movement of the animal, pentobarbitone injection, etc.). Changes in intrauterine pressure smaller than 2 mmHg were not included in the calculations.

Changes in myometrial activity were recorded for 10 h without administration of any hormones.

Fourteen rabbits were used. Five of the "acute" does and 4 of the "chronic" does, all with stable and regular oestrous activity were given 20,000 IU of HCG (Gonadon, LEO, Denmark). The myometrial activity was recorded 1-2 h before the injection and during the subsequent 12 h period. In 10 of the chronic rabbits recordings were also obtained during a period of 3 h on each of the following 10 days. For the determination of concentration of progesterone and oestradiol-17 β in plasma (Batra *et al.* 1978) venous blood samples (2 ml) were collected from the anterior femoral vein before and 2, 4, 8, and 12 h after the injection of HCG. In 4 of the "chronic" rabbits, samples were also obtained at 4 h after the HCG injection.

Results

After administration of hormones the uterine activity followed the typical pattern observed in non-pregnant rabbits (Laudanski *et al.* 1977). The contractions occurred with comparatively high amplitude, low frequency and long duration, and there was distinct relaxation

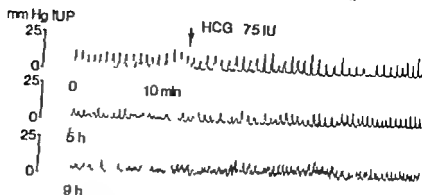


Fig. 1 Effect of HCG injection on the pattern of intrauterine pressure (IUP) recording in a non-pregnant rabbit with a chronically implanted sponge-tipped catheter

phase after each contraction (Fig. 1 2). The uterine activity in the control does not change significantly during the recordings (not shown).

After the injection of HCG a relatively rapid change in the uterine activity occurred. It was visible in some cases as early as 1 h after the injection (not shown) and was conspicuous after 3 h of injection (Fig. 2). The uterine contractions gradually became smaller in amplitude, more frequent, more irregular and a well defined relaxation phase between the contractions could no longer be observed. This type of activity became succeeded by more pronounced with time up to at least 9 h. There were no qualitative differences in the spontaneous activity and in the response to HCG administration between the "acute" and the "chronic" rabbits (Fig. 1 2). In the two "chronic" rabbits recorded after one and two days of HCG injection the myometrial contractility was still depressed.

The plasma progesterone levels before and after the HCG injection are shown in Fig. 3. A maximal increase in progesterone concentration (5.0 ng/ml) occurred already in the first sample taken at 2 h after the injection. The levels gradually decreased thereafter and the mean concentration in samples taken at 24 h after injection was 0.25 ng/ml (not shown).

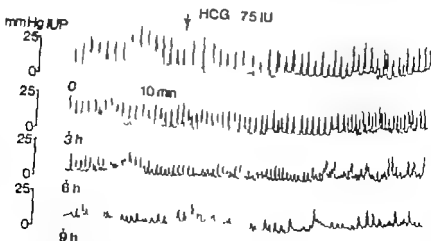
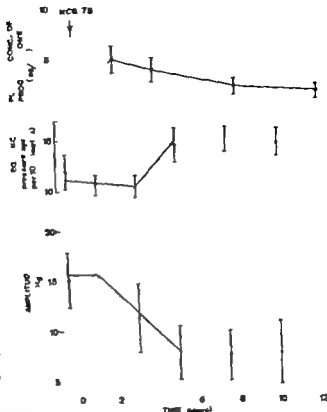


Fig. 2 Effect of HCG on the pattern of IUP in a rabbit recorded by a micro-transducer catheter inserted at an operation 1 h before the recording. The recording pattern and the changes caused by HCG injection were, in general, the same as in the "chronic" rabbits (Fig. 1).



1 Time courses of the changes in plasma concentrations of progesterone and oestradiol and activity of uterine contractions after HCG injection. Values are mean \pm S.E. of 7 to 9 animals. The S.E. of the regression mean value of progesterone is included within the error bars.

Figure). The HCG injection caused no significant change in the plasma oestradiol concentration which ranged between 12 to 64 pg/ml in all samples analysed.

The change in the contractile activity pattern after HCG injection seemed to follow the change in progesterone concentration in plasma (Fig. 3). The amplitude of contraction began to decrease 1 h after the HCG injection and the frequency began to increase after 3 h. After about 5 h these changes were fully developed. This pattern of myometrial activity, i.e. high frequency and low amplitude of contractions, was maintained during the rest of the observation period in spite of a steady decrease in the peripheral plasma progesterone concentrations following the peak value after 2 h of HCG injection.

Myometrial tissue concentration of progesterone (Batra and Bengtsson 1976) was measured in 4 rabbits 12 h after HCG injection. The mean progesterone concentration was 15.7 \pm 1.1 ng/g (range 11.5–17.1).

Discussion

In commonly recording the rabbit myometrial activity under conditions where concentrations of progesterone were changing drastically but within physiological limits, we were able to delineate the effects of this hormone on the myometrial activity. At about 5 h after HCG injection a distinct change, indicative of a shift from non-pregnant to pregnant

type (Laudanski *et al* 1977) in the pattern of myometrial activity was observed. The plasma progesterone concentration was already maximal at the first sampling, 2 h after HCG injection and thereafter decreased gradually. The peak progesterone concentration may have occurred even earlier than after 2 h but the majority of the published data indicate that the maximal plasma progesterone concentration is attained between 2 and 4 h after HCG administration (Hilliard 1973). These observations together with our recent data showing the effects of infusion of progesterone itself on the myometrial activity (Laudanski *et al* 1978) support the contention that progesterone was the major factor responsible for the shift in the pattern of myometrial activity.

The possibility of a direct effect of HCG on the myometrium seems unlikely since one would expect the manifestation of this not only immediately but also short-lived after a single i.v. injection. Dessarzin and Stamm (1962) reported that the administration of HCG was able to inhibit the myometrial activity to the pregnant rat *in situ*. This inhibitory effect was observed within 1 h after an intramuscular injection of HCG. A comparison of the present results with those published by these authors is difficult due to the species difference and to the fact that they used pregnant animals, whereas we studied the effect in non-pregnant ones.

Although the concentration of 20 α -dihydroprogesterone after HCG administration shows a dramatic increase, more than 10 times that of the progesterone concentration, we are aware of any report on the effect of this progestin on the myometrial activity *in situ*. From the present observations one could deduce at least that the effect of 20 α -dihydroprogesterone could not be opposite to that of progesterone, since in that event it would have counteracted the effect of progesterone. The extent to which 20 α -dihydroprogesterone might have reinforced the effect of progesterone by acting synergistically cannot be evaluated until the effects of this steroid individually and in combination with other steroids are known. Preliminary experiments indicate (unpublished) that 20 α -dihydroprogesterone in related high doses had no or slight inhibitory effect on the rabbit myometrial activity.

Schofield (1955) who studied rabbit myometrial activity *in situ* found no immediate effect of progesterone given i.v. and i.m. and came to the conclusion as did Csapo and Lloyd-Jacob (1961) and Porter (1974) that progesterone has a latency in its action on the myometrium. However, these enormous doses of progesterone, together with the vehicles used, could even have had a toxic effect, and interestingly Schofield (1955) reported that the rabbits often died within 30 min of injection of the vehicle, propylene glycol (see also Kälé 1977, Luukkainen and Csapo 1961).

The latency in the effect of progesterone on myometrial activity even after i.v. administration reported in the literature (Schofield 1955, Csapo and Lloyd-Jacob 1961, Porter 1974) seems somewhat paradoxical in view of the fact that steroid hormones are considered to act by altering ion permeability or/and conductance characteristics of the myometrial cell membrane (Abe 1970, Kao 1977, Batra and Bengtsson 1978). The present results showing an almost immediate effect after an elevation of progesterone in plasma together with the data demonstrating a rapid change in myometrial activity after luteolysis in pseudopregnant rabbits (Laudanski *et al* 1978) clearly contradict the theory of a latency in progesterone effect. The latency might have resulted from the use of chronic type rather than the acute

of rabbits in studies reporting this phenomenon. The inflammatory reaction, intra-uterine infection, adhesions etc., which frequently will occur in rabbits with chronically inserted catheters, could significantly affect the intramural diffusion and passage of the injected hormone. However the fact that both "acute" and "chronic" rabbits in the last study responded to HCG treatment in a similar manner cannot easily be reconciled with this explanation.

In conclusion, by using a relatively novel approach we were able to demonstrate that an increase in progesterone levels by increased ovarian output under physiological conditions has a pronounced and immediate depressing effect on the character of myometrial activity.

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References

1. A. Y. The hormonal control and the effects of drugs and ions on the electrical and mechanical activity of the uterus. In: *Symposium on the Uterus*, Eds. E. Billbring, A. S. Breding, A. W. Jones and T. Tomika, 1970, pp. 477. Edward Arnold Publ., London.
2. J. L. P. B. BENTSSON and U. ULSTRUP, Recording of myometrial activity in the non-pregnant rabbit by microtransducer catheter. *Acta Obstet. Gynecol. Scand.* 1978. In press.
3. J. L. P. B. BENTSSON, Effects of diethylstilboestrol and oestrogen extracts on the contractile responses of uterine smooth muscle in the rabbit. *J. Physiol. (Lond.)* 1978. 276: 329-342.
4. J. L. P. B. BENTSSON, A highly efficient method for the extraction of progesterone from uterine tissue. *J. Steroid Biochem.* 1976. 7: 599-603.
5. J. L. P. B. BENTSSON and G. THORSTEN, Estrogen and progesterone concentrations in the rabbit uterus after steroid administration. *Endocrinology* 1978. 102: 268-272.
6. J. L. P. B. BENTSSON, The sponge-tipped catheter—a modification of the open end catheter for recording of myometrial activity in the rabbit. *J. Reprod. Fert.* 1968. 16: 115-118.
7. J. L. P. B. BENTSSON and M. A. LLOYD-JACOB, Effect of progesterone on pregnancy. *Nature (Lond.)* 1961. 192: 124-126.
8. J. L. P. B. BENTSSON and O. B. B. BENTSSON, Action inhibitrice des Gonadotrophes Chorioniques sur l'utérus. *Gynecologie* 1978. 154: 27-36.
9. J. L. P. B. BENTSSON, Corpus luteum function in guinea pigs, hamsters, rats, mice and rabbits. *Biol. Reprod.* 1977. 17: 283-291.
10. A. C. Y. Electrophysiological properties of the uterine smooth muscle. In: *Biology of the Uterus*, Ed. J. L. P. B. BENTSSON, 1977. 424-496. Martin Press, New York and London.
11. J. L. P. B. BENTSSON, M. ÅBERG and S. BÄCKSTRÖM, Differences in the effects of vasopressin and oxytocin on myometrial activity and the possible mediation of prostaglandins. *J. Reprod. Fert.* 1977. 31: 355-364.
12. J. L. P. B. BENTSSON and S. BÄCKSTRÖM, Prostaglandin-induced luteolysis in pregnant and pseudo-pregnant rabbits and the resultant effect on the myometrial activity. *J. Reprod. Fert.* 1978. In press.
13. J. L. P. B. BENTSSON and A. CARL, Effect of phospholipid factor on the activity of progesterone. *Nature (Lond.)* 1978. 272: 331.
14. J. L. P. B. BENTSSON and K. S. B. BENTSSON, The synthesis and secretion of progesterone and 20 α -hydroxyprogesterone by the rabbit ovary in various intervals after single injection of HCG (1). *J. Steroid Biochem.* 1978. 10: 61-67.
15. J. L. P. B. BENTSSON, The local effect of intra-uterine progesterone treatment on myometrial activity in rabbits. *J. Reprod. Fert.* 1968. 15: 437-443.
16. J. L. P. B. BENTSSON, Inhibition of myometrial activity in the pregnant rabbit: evidence for "new" factor. *J. Reprod. Fert.* 1974. 10: 54-61.
17. J. L. P. B. BENTSSON, The influence of the ovarian hormones on myometrial behaviour in the intact rabbit. *J. Physiol. (Lond.)* 1975. 129: 289-304.

Effect of plasma H^+ ion concentration on pancreatic HCO_3^- secretion

By

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Abstract

RÆDER, M. A. MO and S. AUNE. Effect of plasma H^+ -ion concentration on pancreatic HCO_3^- secretion. *Acta physiol. scand.* 1979 105 420-427

The relationship between the rate of pancreatic HCO_3^- secretion and plasma H^+ -ion concentration was investigated in 13 pentothal anesthetized, secretin infused pigs (1.8 C.U./kg b.w. h^{-1} intravenously) during acute metabolic and respiratory acid-base disturbances. Pancreatic HCO_3^- secretion increased to $196 \pm 10\%$ of control during alkalosis and fell to $41 \pm 4\%$ of control during acidosis. Partial metabolic compensation of respiratory acidosis restored HCO_3^- secretion to $87 \pm 6\%$ of control. A proportional relationship was found between HCO_3^- secretion and plasma pH. Different, proportional relationships were found between HCO_3^- secretion and plasma HCO_3^- concentration during metabolic and respiratory acid-base changes. HCO_3^- secretion was independent of H^+ -ion concentration in pancreatic juice. Plasma H^+ -ion concentration, therefore, seems to determine the rate of pancreatic HCO_3^- secretion. This finding supports the hypothesis that a proton pump is responsible for pancreatic HCO_3^- secretion.

Key words: Acid-base back-leakage hypothesis pigs, proton pump, secretin

Some 90% of bicarbonate secreted by the pancreas derives from plasma bicarbonate (Case *et al* 1970). Correspondingly the rate of pancreatic bicarbonate secretion is sensitive to changes in systemic acid-base balance. Previous studies on artificially perfused pancreases have indicated that the rate of secretion of bicarbonate varies in proportion to the bicarbonate content of the perfusate. (Case *et al* 1970, Schulz 1972). Other investigators, however, claim additive effects of perfusate pH and bicarbonate concentration on the secretion rate (Swanson and Solomon 1975). Since bicarbonate secretion implies equimolar transport of H^+ ions into interstitial fluid by secretory cells, a simple hypothesis would be that variations in rate of pancreatic bicarbonate secretion during acid-base changes were due to varying back leakage of H^+ -ions into secretory cells. If so, extracellular H^+ ion concentration would determine the rate of bicarbonate secretion from the pancreas. The first purpose of the present study therefore, was to examine what relationship pertains between plasma H^+ -ion concentration and rate of pancreatic bicarbonate secretion in the intact animal.

The effects of varying plasma H^+ -ion concentration on the rate of pancreatic bicarbonate secretion were examined at normal and high arterial P_{CO_2} in anesthetized pigs during laparotomy and continuous i.v. infusions of secretin. By increasing arterial P_{CO_2} , arterial pH may be lowered while maintaining arterial bicarbonate concentration. If arterial H^+ -ion concentration determines the rate of pancreatic bicarbonate secretion, pancreatic bicarbonate output would parallel arterial pH. Alternatively, were arterial bicarbonate concentration determinant of the rate of pancreatic bicarbonate secretion, bicarbonate output would isoproportionally with arterial bicarbonate concentration at normal and high arterial

The second purpose of the present study was to examine the effects on bicarbonate secretion of increasing H^+ -ion concentration in pancreatic juice. At high P_{CO_2} , CO_2 permeates pancreatic duct epithelium, hydrates in the lumen and raises H^+ -ion concentration in pancreatic juice. If, as maintained by some authorities, pancreatic bicarbonate secretion is a consequence of active H^+ -ion transport across the luminal cell membrane into bicarbonate secreting cells (Schulz *et al.* 1971, Schulz 1972, Simon and Kather 1975, Simon and Kather 1976, Witzmann and Schulz 1973), the raising of luminal H^+ -ion concentration will be expected to enhance the rate of bicarbonate secretion due to reduced concentration difference against which the H^+ -ions have to be transported. Alternatively, if active H^+ -ion transport were confined to the interstitial cell membrane of bicarbonate secreting cells, alterations in luminal H^+ -ion concentration would be expected to have no effect on H^+ -ion transport rate, because the luminal H^+ -ion concentration changes would be fully segregated from the active transport site.

It is found that during constant i.v. secretin infusion, arterial pH determined the rate of pancreatic bicarbonate secretion at normal as well as high arterial P_{CO_2} . Different, proportional relationships were found between the rate of bicarbonate secretion and plasma bicarbonate concentration at normal and high arterial P_{CO_2} . Pancreatic juice H^+ -ion concentration more than doubled during high P_{CO_2} . At comparable plasma pH, however, there was no difference in rates of pancreatic bicarbonate secretion during normal and high P_{CO_2} . Our findings are compatible with the hypothesis that pancreatic bicarbonate secretion is a consequence of active transport of H^+ -ions across the interstitial cell membrane of bicarbonate secreting cells in the pancreas.

Methods

Experiments were carried out on young pigs, weighing 20–25 kg, under general peritoneal anaesthesia (1% 1,1,1-trichloro-2,2,2-fluoroethane) and supplementary doses were given as required, during the experiments. An endotracheal intubation was performed. The animals were artificially ventilated by breathing air/oxygen mixture at 4 l/min, CO_2 being added to the gas mixture, when desired, to increase arterial P_{CO_2} . Anaesthesia was maintained by i.v. periodic administration of pentobarbitone bromide (Pervanol). The carotid vein was cannulated for arterial blood sampling and blood pressure monitoring by means of a catheter (F23GV). The jugular vein was cannulated for infusions. The abdomen was opened by a midline incision. The main pancreatic duct was cannulated by a soft, thin polyethylene catheter (F23GV) for timed collection of pancreatic secretion. The gallbladder and urinary bladder were externally drained by indwelling Foley catheters. The common bile duct was ligated immediately after the dissection, and the pylorus was closed by a stout silk suture. The vaginal innervation of the stomach was left intact. Following insertion of catheters, the abdominal incision was closed by towel clips.

An electric pillow placed under the animal, ensured constant body temperature during the experiments. 800-1000 ml of 0.9% NaCl was infused i.v. during the surgical procedure to expand extracellular fluid volume and maintain good peripheral circulation. Continuous stimulation of pancreatic secretion was ensured during i.v. infusion of secretin (from the Karolinska Institute, Stockholm, Sweden) at 1.8 C.U./kg h. This gave a maximal secretion of HCO_3^- in the pig.

During steady state two 4 min collections of pancreatic juice were made. These samples are referred to as control. Arterial blood specimens were drawn in the middle of each control period for electrolyte and blood gas analysis. Following timed collection of pancreatic juice, 300 μl of pancreatic juice was collected anaerobically for immediate H^+ -ion and Pco_2 determination.

Respiratory acidosis (hypercapnia) was induced by adding CO_2 to the inspired air mixture aiming at an arterial Pco_2 of 100-150 mmHg. During steady state, timed samples of pancreatic juice and blood specimens were obtained as above for analysis (14 animals).

Compensated respiratory acidosis was obtained through i.v. infusion of isotonic NaHCO_3 (4-25 mmol over 30 min) during sustained elevation of arterial Pco_2 . Several pancreatic juice and blood samples were obtained as above during back titration of arterial pH with NaHCO_3 (5 animals).

Acidotic acidosis was induced by i.v. infusion of 100-150 mmol HCl, dissolved in 1000 ml H_2O , over 30 min. Pancreatic juice and blood specimens were obtained as above. Normal acid-base balance was subsequently restored by infusion of 4.2% NaHCO_3 with frequent monitoring of arterial pH and blood gas parameters. A new set of pancreatic juice and blood samples was obtained upon regaining normal acid balance and included in the control group (8 animals).

Metabolic alkalosis was obtained through i.v. infusion of 450-550 mmol isotonic NaHCO_3 over 180 min. Blood specimens and pancreatic juice samples were obtained as above (6 animals). All animals were killed at the end of the experiments.

Pancreatic juice and plasma H^+ -ion concentrations and Pco_2 were determined by an Instrumental Laboratories Blood Gas Analyzer 313 (Lexington, Mass., U.S.A.). Plasma HCO_3^- concentration was calculated according to Henderson-Hasselbalch's formula: $\text{HCO}_3^- \text{ concentration} = \text{antilog}(\text{pH} - 6.1) \times 0.03 \times \text{Pco}_2$. Pancreatic juice HCO_3^- concentration was estimated as the difference between the sum of Na^+ and K^+ concentrations and Cl^- concentration in pancreatic juice. Plasma and pancreatic juice Na^+ and Cl^- concentrations were measured by an Instrumental Laboratories 343 flame photometer. Chloride determinations were made with a CM 10 chloride titrator (Radiometer Copenhagen, Denmark).

Statistical methods

Wilcoxon's signed rank test for paired samples and Wilcoxon's rank test for unpaired measurements (Mann-Whitney test) were used to evaluate differences between observations (Snedecor 1967). Linear regression were calculated using the method of least squares fit. Multiple regression was calculated using stepwise variables (Snedecor 1967).

Results

Changes in systemic acid base balance had pronounced effects on pancreatic bicarbonate secretion (Table I). During respiratory acidosis, pancreatic bicarbonate secretion fell by an average of $66 \pm 6\%$. During metabolic acidosis, pancreatic bicarbonate secretion fell by an average of $49 \pm 6\%$. Metabolic alkalosis greatly increased pancreatic bicarbonate secretion. On average, secretion doubled during NaHCO_3 infusion at normal Pco_2 . At high Pco_2 NaHCO_3 infusion restored plasma H^+ -ion concentration and pancreatic bicarbonate secretion towards control.

Effect of plasma H^+ -ion concentration on pancreatic HCO_3^- secretion

The rate of pancreatic bicarbonate secretion increased with plasma pH during normal and high Pco_2 . Fig. 1 indicates a linear relationship between the rate of secretion and plasma pH, the correlation coefficient being 0.82. Pancreatic bicarbonate secretion was measured down to pH 6.95—secretion could be halted by allowing plasma pH to fall below this value.

Arterial concentrations			Pancreatic juice concentrations			Pancreatic secretions					
pH	HCO ₃ mmol/l	Prox. mmol/g	pH	HCO ₃ mmol/l	Prox. mmol/g	Na mmol/l	K mmol/l	Cl ⁻ mmol/l	Volume ml/ml	CO ⁻ mmol/min	HCO ₃ ⁻
Control	7.38 ± 0.01	41.4 ± 0.6	41 ± 1	6 ± 0	33 ± 1	156 ± 1	4.9 ± 0.1	14 ± 1	1.18 ± 0.07	174 ± 11	100 ± 3
Respiratory acidosis	7.10 ± 0.03	33.7 ± 2.3	33.8 ± 3	27 ± 2	130 ± 7	159 ± 8	4.7 ± 0.4	24 ± 3	0.43 ± 0.01	63 ± 19	34 ± 6
Metabolic acidosis	7.09 ± 0.03	14.8 ± 1.0	43 ± 2	10 ± 3	43 ± 3	146 ± 1	5.6 ± 0.3	21 ± 2	0.66 ± 0.08	89 ± 12	51 ± 6
Metabolic alkalosis	7.26 ± 0.02	39.3 ± 2.3	41 ± 2	6 ± 0	37 ± 2	162 ± 2	6.3 ± 0.3	7 ± 1	2.40 ± 0.13	385 ± 22	196 ± 10
Overpackaged respiratory acidosis	7.14 ± 0.04	29.9 ± 3.5	33.1 ± 4	21 ± 1	114 ± 12	166 ± 2	4.1 ± 0.2	10 ± 2	1.14 ± 0.12	186 ± 22	87 ± 6

Data presented are mean values \pm S.E. from 15 expts. on anesthetized young pigs (20-25 kg b.w.). Control arterial plasma H^+ -ion concentration at normal Pa CO_2 respiratory acidosis = addition of CO_2 to inspired air/ O_2 mixture. Metabolic acidosis = 100-150 mmol/l infusion of 100-150 mmol/l over 30-40 min. Metabolic alkalosis = infusion of 1.5-2 liter 1.2% NaHCO_3 over 30-40 min. Overpackaged respiratory acidosis = following infusion of 1.0-1.5 liter 1.2% NaHCO_3 over 30-40 min. " per cent of value during control, # number of analyzed pancreatic juice samples.

An electric pillow placed under the animal, ensured constant body temperature during the exps. 100-150 ml of 0.9% NaCl was infused i.v. during the surgical procedure to expand extracellular fluid volume and maintain good peripheral circulation. Continuous stimulation of pancreatic secretion as reported from i.v. infusion of secretin (from the Karolinska Institute, Stockholm, Sweden) at 1.2 U/kg b.w. 1-2 ml gave a maximal secretion of HCO_3^- in the pig.

During steady state two 4 ml collections of pancreatic juice were made. These samples are referred to as control. Arterial blood specimens were drawn in the middle of each control period for electrolyte and blood gas analysis. Following timed collection of pancreatic juice 300 μl of pancreatic juice was collected anaerobically for immediate H^+ -ion and PCO_2 determination.

Respiratory acidosis (hypercapnia) was induced by adding CO_2 to the inspired air/oxygen mixture aiming at an arterial PCO_2 of 100-150 mmHg. During steady state, timed samples of pancreatic juice and blood specimens were obtained as above for analysis (14 animals).

Compensated respiratory acidosis was obtained through i.v. infusion of isotonic NaHCO_3 (1.2-2.0 mmol/over 30 min) during sustained elevation of arterial PCO_2 . Several pancreatic juice and blood samples were obtained as above, during back titration of arterial pH with NaHCO_3 (5 animals).

Acidotic acidosis was induced by i.v. infusion of 100-150 mmol HCl, dissolved in 1000 ml H_2O , in 30 min. Pancreatic juice and blood specimens were obtained as above. Normal acid-base balance was subsequently restored by infusion of 4-6 mmol NaHCO_3 with frequent monitoring of arterial pH and blood gas parameters. A new set of pancreatic juice and blood samples was obtained upon regaining normal acid-base balance and included in the control group (8 animals).

Metabolic alkalosis was obtained through i.v. infusion of 250-350 mmol isotonic NaHCO_3 over 30 min. Blood specimens and pancreatic juice samples were obtained as above (6 animals). All animals were killed at the end of the exps.

Pancreatic juice and plasma H^+ -ion concentrations and PCO_2 were determined by an Instrumental Laboratories Blood Gas Analyzer 313 (Lexington, Mass., U.S.A.). Plasma HCO_3^- concentration was calculated according to Henderson-Hasselbalch's formula HCO_3^- concentration = $\text{antilog}(\text{pH} - 6.1) \times 10^{\text{pH}}$. Pancreatic juice HCO_3^- concentration was estimated as the difference between the sum of Na^+ and K^+ concentrations and Cl^- concentration in pancreatic juice. Plasma and pancreatic juice Na^+ and K^+ concentrations were measured by an Instrumental Laboratories 343 flame photometer. Chloride determinations were made with a CM 10 chloride titrator (Radiometer Copenhagen, Denmark).

Statistical methods

Wilcoxon's signed rank test for paired samples and Wilcoxon's rank test for unpaired measurements (Mann-Whitney test) were used to evaluate differences between observations (Snedecor 1967). Linear regressions were calculated using the method of least squares fit. Multiple regression was calculated using orthogonal variables (Snedecor 1967).

Results

Changes in systemic acid-base balance had pronounced effects on pancreatic bicarbonate secretion (Table I). During respiratory acidosis, pancreatic bicarbonate secretion fell by an average of $66 \pm 6\%$. During metabolic acidosis, pancreatic bicarbonate secretion fell by an average of $49 \pm 6\%$. Metabolic alkalosis greatly increased pancreatic bicarbonate secretion. On average, secretion doubled during NaHCO_3 infusion at normal PCO_2 . At high PCO_2 NaHCO_3 infusion restored plasma H^+ -ion concentration and pancreatic bicarbonate secretion towards control.

Effect of plasma H^+ -ion concentration on pancreatic HCO_3^- secretion

The rate of pancreatic bicarbonate secretion increased with plasma pH during normal and high PCO_2 . Fig. 1 indicates a linear relationship between the rate of secretion and plasma pH, the correlation coefficient being 0.82. Pancreatic bicarbonate secretion was maximum down to pH 6.95—secretion could be halted by allowing plasma pH to fall below this value.

however the rate of pancreatic bicarbonate secretion was significantly lower at any one HCO_3^- concentration during hypercapnia than during normocapnia. Thus, at plasma pH concentration of 23 mmol/l, pancreatic bicarbonate secretion averaged 103% during normocapnia and 16% during hypercapnia.

Effect of plasma P_{CO_2} on pancreatic juice composition

Hypercapnia increased pancreatic juice P_{CO_2} and H^+ -ion concentration (Table I). During normocapnia, the juice H^+ -ion concentration ranged between 5–10 nmol/l and remained totally independent of plasma H^+ -ion concentration. Hypercapnia increased juice H^+ -ion concentration to 23 ± 1 nmol/l ($p < 0.05$). The increase in the juice H^+ -ion concentration was paralleled by an increase in P_{CO_2} in pancreatic juice from 41 ± 1 mmHg to 149 ± 3 mmHg. However despite this increase P_{CO_2} in pancreatic juice was still 27 ± 4 mmHg below arterial P_{CO_2} .

There was no consistent relationship between the rate of pancreatic bicarbonate secretion and pancreatic juice P_{CO_2} . Similarly the rate of bicarbonate secretion exhibited no consistent relationship to H^+ -ion concentration in pancreatic juice.

Discussion

The present study demonstrates that plasma H^+ -ion concentration determines the rate of pancreatic bicarbonate secretion in the intact animal. Previous studies on isolated organ preparations have indicated that the secretion rate is proportional to the perfusate HCO_3^- concentration (Case *et al* 1970; Schulz 1972) and that the perfusate pH and the HCO_3^- concentration have additive effects on the secretion rate (Swanson and Solomon 1975). However by relating the rate of bicarbonate secretion to plasma pH and HCO_3^- concentration during normocapnia and hypercapnia, it was possible, in the present study to dissociate the effects of plasma H^+ -ion and plasma HCO_3^- concentration on the bicarbonate secretory mechanism. We found that alterations in the secretion rate during variations in plasma P_{CO_2} concentration could be accounted for by the concomitant changes in plasma pH . Therefore, at normal and high P_{CO_2} , plasma pH and hence interstitial fluid pH , determines the rate of pancreatic bicarbonate secretion.

Asymmetry of sensitivity of the bicarbonate secretory mechanism to extracellular H^+ was demonstrated: there was no definite effect on bicarbonate transport of increasing extracellular H^+ -ion concentration in pancreatic ducts, as during high P_{CO_2} , while changes in H^+ -ion concentration on the interstitial side of the secretory cells elicited large changes in the rate of bicarbonate secretion. Since bicarbonate secretion implies equimolar transport of H^+ ions across the interstitial cell membrane of bicarbonate secreting cells, a simple explanation of the asymmetric sensitivity of the secretory mechanism to extracellular H^+ ions could be that interstitial H^+ -ions interfere with the activity of a proton pump located on or in the interstitial cell membrane.

There are several ways in which interstitial H^+ -ions might impair the activity of a proton pump. One hypothesis would be that interstitial H^+ -ions regulate the net transport rate by moving back through the interstitial cell membrane into bicarbonate secreting cells. The

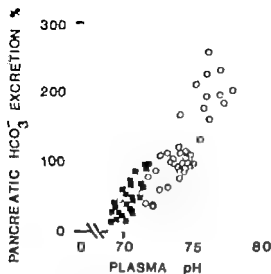


Fig. 1

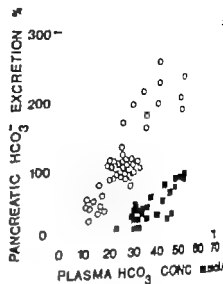


Fig. 2

Fig. 1 Relationship between rate of pancreatic HCO_3^- secretion and plasma pH during acute changes in systemic acid-base balance. O arterial $P_{\text{CO}_2} = 42 \pm 1$ mmHg. ■ arterial $P_{\text{CO}_2} = 145 \pm 3$ mmHg. HCO_3^- secretion is calculated as per cent of control. For definitions of acid-base disturbances, see footnote Table I. Secretin was infused 1 ± 1.8 C.U./kg b.wt. h. ■ number of expts. = 15.

Fig. 2 Relationship between pancreatic HCO_3^- secretion and plasma HCO_3^- concentration during changes in systemic acid-base balance. Symbols and definitions as in Fig. 1. Number of expts. = 15.

through administration of high P_{CO_2} in the respiratory gas mixture. This effect of plasma P_{CO_2} on secretion was reversible, as is evident by the return of secretion to control values at restoration of normal plasma pH whether this was achieved through normalization of plasma P_{CO_2} or through intravenous infusion of NaHCO_3 (Table I).

Multiple regression analysis of the effect of plasma pH and P_{CO_2} on the rate of pancreatic bicarbonate secretion at plasma pH < control yielded the following equation.

$$\text{Rate of } \text{HCO}_3^- \text{ secretion} = 1074.54 + 157.49 \text{ pH} + 0.054 P_{\text{CO}_2} \text{ S.E.} = \pm 151$$

If P_{CO_2} is omitted the equation becomes

$$\text{Rate of } \text{HCO}_3^- \text{ secretion} = -1006.75 + 148.64 \text{ pH S.E.} = \pm 152$$

Thus, the standard error of a multiple regression analysis, including plasma P_{CO_2} is nearly the same as the standard error of a simple regression calculated for plasma pH on rate of pancreatic bicarbonate secretion. This indicates that plasma P_{CO_2} is not an important determinant of pancreatic bicarbonate secretion under the conditions of normal and near normal plasma P_{CO_2} and H^+ ion concentrations.

Relationships between plasma HCO_3^- concentration and pancreatic bicarbonate

Pancreatic bicarbonate secretion increased in proportion with plasma bicarbonate concentration at normal and high P_{CO_2} (Fig. 2) the correlation coefficients being 0.84 and 0.89 respectively.

lower, the rate of pancreatic bicarbonate secretion was significantly lower at any HCO_3^- concentration during hypercapnia than during normocapnia. Thus, at plasma HCO_3^- concentration of 25 mmol/l, pancreatic bicarbonate secretion averaged 103% during normocapnia and 16% during hypercapnia.

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Hypercapnia increased pancreatic juice P_{CO_2} and H^+ -ion concentration (Table I). During normocapnia, the juice H^+ -ion concentration ranged between 5–10 nmol/l and remained nearly independent of plasma H^+ -ion concentration. Hypercapnia increased juice H^+ -ion concentration to 23 ± 1 nmol/l ($p < 0.05$). The increase in the juice H^+ -ion concentration was paralleled by an increase in P_{CO_2} in pancreatic juice from 41 ± 1 mmHg to 149 ± 3 mmHg. However despite this increase P_{CO_2} in pancreatic juice was still $\sim 7 \pm 4$ mmHg below arterial P_{CO_2} .

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The present study demonstrates that plasma H^+ -ion concentration determines the rate of pancreatic bicarbonate secretion in the intact animal. Previous studies on isolated organ preparations have indicated that the secretion rate is proportional to the perfusate HCO_3^- concentration (Cise *et al.* 1970, Scholz 1972) and that the perfusate pH and the HCO_3^- concentration have additive effects on the secretion rate (Swanson and Solomon 1975). However, by relating the rate of bicarbonate secretion to plasma pH and HCO_3^- concentration during normocapnia and hypercapnia, it was possible, in the present study to dissociate the effects of plasma H^+ -ion and plasma HCO_3^- concentration on the bicarbonate secretory mechanism. We found that alterations in the secretion rate during variations in plasma HCO_3^- concentration could be accounted for by the concomitant changes in plasma pH. Therefore, at normal and high P_{CO_2} , plasma pH and hence interstitial fluid pH, determines the rate of pancreatic bicarbonate secretion.

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There are several ways in which interstitial H^+ -ions might impair the activity of a proton pump. One hypothesis would be that interstitial H^+ -ions regulate the net transport rate by moving back through the interstitial cell membrane into bicarbonate secreting cells. The

simplest concept would be that back leakage of H^+ ions would occur through leak pathways of fixed proton conductance. Accordingly the rate of back-leakage would be expected to be proportional to plasma H^+ -ion concentration. The results of the present investigation indicate a more complex relationship between the plasma H^+ -ion concentration and the rate of back leakage, and therefore argue against such a simplified model. Alternatively H^+ ions may leak back through the cell membrane by combining with a membrane bound carrier. Such a carrier mediated diffusion would be expected to approach a transport maximum as interstitial H^+ ion concentration increases. The observed plasma pH effect on secretion in the present study may be interpreted within this framework. Assuming that there is a constant rate of H^+ ion secretion during constant secretin infusion, the observed diminution in rate of bicarbonate secretion and hence net H^+ -ion transport rate, could be due to carrier mediated back-leakage of H^+ ions. Leakage would be proportional to plasma H^+ ion concentration during alkalosis and would become progressively less dependent on plasma H^+ -ion concentration at normal plasma pH and during acidosis, due to membrane carrier saturation with H^+ -ions.

The possibility remains that external H^+ ions may interfere with proton pump activity through an inhibitory action on oxidative metabolism coupled to active H^+ -ion transport. This effect of external H^+ ions on proton pumps has been claimed to exist in other tissues (Beauwens and Al-Awqati 1976). There are no available data on pancreatic metabolic rate during acid base disturbances, to either refute or validate such a hypothesis.

It is of interest to note how the findings of the present study otherwise parallel the behaviour of the proton pump in turtle bladder epithelium. Turtle bladder epithelium possesses similar asymmetry in sensitivity to external H^+ -ions: an increase in H^+ -ion concentration on the serosal side of the epithelium has no effect on H^+ -ion secretion (Beauwens and Al-Awqati 1976), while an increase in H^+ ion concentration on the mucosal side, which is believed to be the aspect of the cell where the proton pump is located, depresses the rate of H^+ ion secretion (Beauwens and Al-Awqati 1976, Schwartz *et al.* 1972, Stelmets 1967).

Currently there are two main hypotheses on the cellular mechanism of pancreatic bicarbonate secretion. The proton pump hypothesis, advanced by Davies (1949), proposes that H^+ ions are secreted into blood from pancreatic cells concurrently HCO_3^- ions pass into the secretory ducts.

The alternative HCO_3^- -ATPase hypothesis is based on the finding of HCO_3^- -ATPase in membrane fragments of pancreatic tissue (Simon *et al.* 1972, Simon and Thomas 1973, Witzmann and Schulz 1973, Witzmann *et al.* 1974). HCO_3^- -ATPase has been suggested to function either as an anion transport mechanism (Schulz *et al.* 1969, Simon *et al.* 1972), or as a proton pump in the luminal cell membrane (Schulz *et al.* 1971, Schulz 1972, Simon and Kather 1976, Witzmann and Schulz 1973). A proton pump in this location would be unusual in the sense that it would actively transport H^+ -ions from the pancreatic duct lumen into ductal epithelial cells. If responsible, HCO_3^- -ATPases would exhibit maximal activity during stimulation under physiological conditions, since *in vitro* studies indicate that maximal enzyme activity is reached at ambient HCO_3^- concentrations of 25 $\mu\text{mol/l}$ (Simon and Thomas 1972). Furthermore, pH sensitivity of this enzyme is not very marked (Simon and Thomas 1972). Therefore, any effects of plasma H^+ ions on such an HCO_3^- -ATPase are

It could necessarily be due to H^+ -ion leakage into pancreatic juice, with varying short-circuiting of the HCO_3^- -ATPase pump.

The findings of the present study argue against this possibility: during high P_{CO_2} pancreatic juice H^+ -ion concentration increased significantly thus reducing the chemical driving force for H^+ -ion back-leakage. However changes in P_{CO_2} per se did not significantly change the rate of pancreatic bicarbonate secretion in our experiments.

In conclusion, it appears that plasma H^+ -ion concentration determines the rate of pancreatic bicarbonate secretion in the intact animal. H^+ -ion concentration in pancreatic juice, however, does not appear to influence bicarbonate output. Asymmetry in effects of environmental H^+ -ions is compatible with the hypothesis of a proton pump in the interstitial cell surface of bicarbonate secreting cells.

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References

- ALPER, R. and Q. AL-AQATI, Active H^+ transport in the turtle urinary bladder. *J. gen. Physiol.* 1976, 68: 43-59.
- ALPER, R. M., T. SCHWARTZ and R. D'A. WYNE, The origin and secretion of pancreatic juice bicarbonate. *J. Physiol. (Lond.)* 1970, 218: 1-12.
- ALPER, R. E., The role of carbon dioxide in the secretion of hydrogen and bicarbonate ions. *J. Physiol. (Lond.)* 1969, 207: 25P.
- ALPER, R., Pancreatic bicarbonate transport I. General secretory. Academic Press, New York and London, 1972.
- ALPER, R. and F. STADLER and K. J. ULLMAN, Lipid soluble weak organic acid buffers as "substrate" for membrane transport. *Pflügers Arch. ges. Physiol.* 1971, 323: 121-140.
- ALPER, R. and A. YAMADA and M. WISSE, Micropuncture studies on the pancreas of the rabbit. *Pflügers Arch. ges. Physiol.* 1969, 328: 277-290.
- ALPER, R. and J. H. S. ROY and P. R. STEINMETZ, Carbonic dehydratase function and the epithelial organization of H^+ secretion in turtle urinary bladder. *J. clin. Invest.* 1972, 51: 2633-2642.
- ALPER, R. and H. KATNER, Neue Aspekte des Bicarbonat-Transporters im exokrinen Pankreas. *Verh. Dtsch. Ges. Path. 1975* 41: 1271-1273.
- ALPER, R. and H. KATNER, Die Bedeutung der HCO_3^- -ATPase in der $\text{H}^+/\text{HCO}_3^-$ Sekretion. *Zell. Wachst. 1975* 34: 91-104.
- ALPER, R. and L. THOMAS, HCO_3^- -stimulated ATPase from mammalian pancreas: properties and its association with other enzyme activities. *Biochim. Biophys. Acta (Amst.)* 1972, 263: 434-442.
- ALPER, R. R. KATNER and G. SACER, The presence of an HCO_3^- -ATPase in pancreatic tissue. *Biochem. biophys. Res. Commun.* 1972, 46: 293-300.
- ALPER, R. and W. G. COCHRAN, *Statistical Methods*. Ames, Iowa, Iowa State Univ. Press, 1967.
- ALPER, R. R. Characteristics of hydrogen ion transport in urinary bladder of star turtle. *J. clin. Invest.* 1971, 50: 1531-1541.
- ALPER, R. C. H. and A. K. SØRENSEN, Micropuncture analysis of the cellular mechanisms of electrolyte secretion by the in vitro rabbit pancreas. *J. gen. Physiol.* 1973, 63: 23-43.
- ALPER, R. and J. SCHWARTZ, Influence of amphotericin, azlonide, ionophores, and 2,4-dinitrophenol on the secretion of the isolated cat pancreas. *Pflügers Arch. ges. Physiol.* 1973, 329: 317-338.
- ALPER, R. and A. L. CHAMBERS, J. W. COCHRAN and J. SCHWARTZ, The distribution of membrane bound enzymes in the acid and duct of the cat pancreas. *Pflügers Arch. ges. Physiol.* 1974, 347: 39-47.

Pregnancy induced alterations in the turnover rate of ^3H noradrenaline formed from ^3H tyrosine in guinea-pig uterus

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Abstract

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The rate of noradrenaline (NA) turnover in the non-pregnant and pregnant guinea-pig uterus was determined after the administration of the labelled precursor tyrosine. Endogenous NA was determined chemically. In non-pregnant animals the turnover of ^3H NA was slower in the uterine horns ($t_{1/2}$ NA and cervix ($t_{1/2}$ = 9 h) than in the reference organ, heart ($t_{1/2}$ = 6 h). During pregnancy (60-65 days) endogenous NA in the heart increased in pace with tissue weight gain, yet, in the uterus there was a drastic reduction, down to zero levels. ^3H NA turnover increased significantly in the cervix, so ^3H formation remained in the uterine horns (a situation resembling that following 6-hydroxydopamine α -sympathectomy of non-pregnant animals), and the turnover was unaltered in the heart. The observations confirm the general idea. Illustrated by the results in the heart, of a functional adaptation with a causal neuroon-target relationship during increased physiological demands. They also confirm that a very local and complex neuroon-target relationship develops in the uterus during pregnancy—involving functional and structural denervation of the adrenergic nerve plexus in the uterine horns, whereas an increased activation might explain the changes in neuronal NA and its turnover in the cervix.

The adrenergic innervation in the uterus of guinea pig and many other species including man undergoes dramatic changes during pregnancy associated near term with a reduction in the level of the noradrenaline (NA) content to near zero values in both the uterine horns and the cervix (Owman *et al* 1975). In the uterine tissue surrounding the fetus (perifetal uterus), the *in vitro* activity of tyrosine hydroxylase (Alm *et al* 1979 a) and the axonal synthesis of ^3H NA (Alm *et al* 1979 b) are lowered to non-detectable levels. These changes seem to reflect a widespread and total peripheral adrenergic degeneration of the adrenergic nerve plexus in the perifetal uterus (Thorbert *et al* 1978 b Spörroing *et al* 1979). In the cervix alterations have been observed in these two parameters (Alm *et al* 1979 a, Alm *et al* 1979 b). A more or less intact nerve plexus was found in this region by the use of α -methyl- ^3H -NA incubations (Thorbert *et al* 1978 b). There is at present no explanation for the cause of this transmitter decay in the cervix (Owman *et al* 1975).

In order to relate the above mentioned changes to a more dynamic aspect of adrenergic action, the formation of ³H NA from ³H-tyrosine was studied in the guinea-pig uterus before and during pregnancy and compared with that following chemical sympathectomy (6-OH-DA).

Materials and Methods

Animals. Female guinea-pigs of mixed strain were used. Sexually mature virgin animals (400–500 g b.wt.) in the oestrous stage, as judged from the closed vagina, were selected. Chemical sympathectomy was performed using small injections of 6-hydroxydopamine with one day interval between each injection (100 µg/kg dissolved in 0.9% saline containing 0.2 mg/ml of ascorbic acid). Animals at 60–65 days of pregnancy were obtained by palpation. After killing under light ether anaesthesia the day of pregnancy was determined by measurement of mean weight and crown-rump length of the fetuses (Drepper 1970, Johnson 1969).

Experimental procedures. A total of 19 animals received 5 µg/kg ³H-tyrosine (—trans-3,5-³H 51 Cl/tyrosine, New England Nuclear) into the jugular vein during halothane anaesthesia (Halothane, Hoechst AG). At various time intervals they were sacrificed and the heart and whole uterus were rapidly dissected out. The uterus was divided into the cervix and uterine horns (only one used for analysis). In the case of prenatally (4 animals) both uterine horns were analyzed separately. The tissues were dried between two sheets of paper, weighed, and homogenized in ice-cold 0.4 N perchloric acid. Aliquots of the tissue were further processed essentially according to Persson and Waldeck (1968) (see also Persson 1970) to separate the ³H-catechol compounds formed and their corresponding ³H-acetylated metabolites on alumina columns. Potassium hydroxide was used instead of potassium carbonate for pH adjustment of the eluates. To get stable recoveries, 50 µg of unlabelled norepinephrine, dopamine, normetanephrine and acetylnorepinephrine were added together to the respective aliquot of homogenate. The ³H-catechol fraction eluted was applied on ion exchange columns (Dowex 50 W X4, diameter 4 mm, length 140 mm, pH 1) to separate the ³H-NA and ³H-DA formed by the differential elution with 1N and 2N HCl (Persson and Waldeck 1968, Persson 1970). The hydrochloric acid eluates of ³H-NA and ³H-DA were put in 10 ml vials and evaporated to dryness. The radioactive residues were taken up in 1 ml ethanol containing 0.1 M HCl, and 9 ml scintillation solution was added (5 g PPO—2.5 diphenylloxazole, and 0.3 g methyl POPOP in 4-iso-2-(4-methyl-5-phenylisoxazolyl)-benzene per liter toluene). Radioactivity was measured in liquid scintillation spectrometer and expressed as dpm per g tissue (specific activity) or as dpm per organ (total activity). Quenching was corrected for using external standardization. Recovery for ³H-NA 15.1% (n = 18) and for ³H-DA 75.57 ± 1.41% (n = 18). No corrections for recovery were made. Detectable ³H-DA was found in any of the different organs investigated.

Tissue extraction. NA in cardiac and uterine tissues was extracted by perchloric acid, separated on cation exchange (Dowex) columns and finally the amount present determined fluorimetrically (Berliner *et al.* 1961, Kilgus 1961).

Statistics. In text and figures, statistical observations are given as arithmetical mean ± S.E., = standard error of the mean. Observed differences in means are evaluated using Student's *t*-test. In the analysis of the percentage of ³H-NA after ³H-tyrosine injection the values for dpm/g were expressed as log₁₀. Regression lines were calculated according to the method of least squares. Conventional statistical methods were used for tests of linearity and slope. Differences in slopes were evaluated according to Pappas (1946). For the constant (b) for the decline in ³H-NA as calculated according to the relationship $k = \text{slope} \cdot \ln 10$. For the half time of ³H-NA as calculated as $(\ln 0.5)/k$ (Goldstein, Aronow and Kalman 1969).

Results

Level of endogenous NA in the various tissues and the amount of ³H-NA formed under various experimental conditions are summarized in Table I.

Heart. Total content of endogenous NA was increased by 66% in pregnant animals compared to virgin controls (*p* < 0.001). This increase was parallel to the weight gain observed in pregnancy and, accordingly no significant difference was seen in the NA con-

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THORBERT G., P ALM CH OWMAN and N-O SJÖSTRÖM *Pregnancy-induced alterations in the turnover rate of ^3H -noradrenaline formed from ^3H tyrosine in guinea-pig uterus. Acta physiol. scand 1979 105: 428-436.*

The rate of noradrenaline (NA) turnover in the non-pregnant and pregnant guinea-pig uterus was determined after the administration of the labelled precursor tyrosine. Endogenous NA was determined fluorimetrically. In non-pregnant animals the turnover of ^3H NA was slower in the uterine horns ($t_{1/2} = 9$ h) and cervix ($t_{1/2} = 9$ h) than in the reference organ, heart ($t_{1/2} = 6$ h). During pregnancy (60-65 days of gestation) endogenous NA in the heart increased in pace with tissue weight gain, yet, in the uterus there was a drastic reduction, down to zero levels. ^3H NA turnover increased significantly in the cervix, no ^3H formation remained in the uterine horn (a situation resembling that following 6-hydroxydopamine beta-sympathectomy of non-pregnant animals), and the turnover was unaltered in the heart. The observations confirm the general idea, illustrated by the results in the heart, of a functional adaptation with a constant neuron-target relationship during increased physiological demands. They also confirm that a very fast and complex neuron-target relationship develops in the uterus during pregnancy—involving functional and structural denervation of the adrenergic nerve plexus in the uterine horns, whereas an increased action might explain the changes in neuronal NA and its turnover in the cervix.

The adrenergic innervation in the uterus of guinea-pig and many other species indeed undergoes dramatic changes during pregnancy associated near term with a reduction in the level of the noradrenaline (NA) content to near zero values in both the uterus and the cervix (Owman *et al.* 1975). In the uterine tissue surrounding the fetus (perifetal uterus), the *in vitro* activity of tyrosine hydroxylase (Alm *et al.* 1979 a) and the axonal uptake of ^3H NA (Alm *et al.* 1979 b) are lowered to non-detectable levels. These changes seem to reflect a widespread and total peripheral adrenergic degeneration of the adrenergic nerve plexus in the perifetal uterus (Thorbert *et al.* 1978 b, Spörngren *et al.* 1979). In the cervix alterations have been observed in these two parameters (Alm *et al.* 1979 a, Alm *et al.* 1979 b). A more or less intact nerve plexus was found in this region by the use of α -methyl-H incubations (Thorbert *et al.* 1978 b). There is at present no explanation for the cause of transmitter decay in the cervix (Owman *et al.* 1975).

TABLE I. Endogenous NA and ³H-NA formed from ³H-tyrosine in uterus and heart under various experimental and reproductive conditions. Values are means \pm S.E. n = number of determinations.

pt	Reproductive state and experimental condition	NA content (μ g/organ)	NA concentration (μ g/g)		³ H-NA formed per organ at 4 h (dpm)	³ H NA formed per g tissue at 4 h (dpm)	
ut	Non-pregnant	2.47 \pm 0.16	2.07 \pm 0.13	6	12 863 \pm 2 250	10 384 \pm 1 969	4
	6-OH DA	0.13 \pm 0.02	0.08 \pm 0.01	5	1 310 \pm 96	1 076 \pm 141	4
	Pregnant	4.10 \pm 0.25	1.81 \pm 0.14	5	35 022 \pm 2 670	13 182 \pm 1 002	4
hr	Non-pregnant	0.28 \pm 0.02	1.40 \pm 0.07	6	302 \pm 251	2 390 \pm 443	4
	6-OH-DA	0.03 \pm 0.00	0.09 \pm 0.01	5	36 \pm 21	196 \pm 113	4
	Pregnant	0.02 \pm 0.01	0.03 \pm 0.01	5	1 879 \pm 249	881 \pm 162	4
trv	Non-pregnant	0.31 \pm 0.02	1.23 \pm 0.11	6	813 \pm 160	2 081 \pm 375	4
	6-OH DA	0.02 \pm 0.00	0.06 \pm 0.01	5	24 \pm 24	128 \pm 128	4
	Pregnant	0.01 \pm 0.00	0.00 \pm 0.00	5	non-detectable	non-detectable	4

at and non-pregnant animals (Table II). In agreement with this, the apparent half-lives for turnover were very similar in the hearts of the two groups: 5.7 h in non-pregnant and 5.9 h in pregnant animals. The turnover rate in the heart of non-pregnant animals was faster than in the cervix ($p = 0.10$) and uterine horn ($p < 0.05$).

At term pregnancy the total amount of endogenous NA was reduced to 7% compared to non-pregnant animals. Due to weight gain of the organ during pregnancy the reduction in tissue concentration of endogenous NA was even more pronounced, being 1/2 of controls. At term pregnancy the total amount of ³H NA formed had clearly risen ($p = 0.01$), whereas the specific activity of ³H NA was reduced to one third ($p < 0.01$) compared to non-pregnant animals. Parallel reduction in endogenous NA and in ³H-NA formed was seen after 6-OH DA injection.

The highest figures for ³H-NA formed from ³H-tyrosine were seen at 4 h in term pregnancy and at 1 h in the non-pregnant animals. From these peak values the tissue ³H-NA crossed. The reduction in log total tissue ³H NA activity was linear with regard to time in non-pregnant ($p = 0.025$) and non-pregnant ($p = 0.05$) animals. The negative slope was significantly different from zero in pregnant ($p < 0.001$) as well as non-pregnant animals ($p = 0.005$). However the magnitude of this negative slope was almost 3 times greater in the pregnant animals (Table II) and, accordingly the apparent half-lives for the turnover was shorter: about 4 h. This difference in slope was significant ($p < 0.01$).

TABLE II. Values for slope (k) and apparent half-life ($t_{1/2}$) of transmitter turnover based on regression analysis of changes in tissue ³H-NA after ³H-tyrosine injection. Statistical evaluation of differences in k -values in non-pregnant (NP) and pregnant (P) animals, n.s. = non significant.

	Reproductive state	$k \pm$ S.D.	$t_{1/2}$ (h)	$k_{NP} + k_P$
cervix	Non-pregnant	0.121 \pm 0.024	5.7	n.s.
	Pregnant	0.117 \pm 0.043	5.9	
uterus	Non-pregnant	0.077 \pm 0.020	9.1	$p = 0.01$
	Pregnant	-0.185 \pm 0.033	3.7	
uterine horn	Non-pregnant	0.067 \pm 0.020	10.4	—
	Pregnant	0	—	

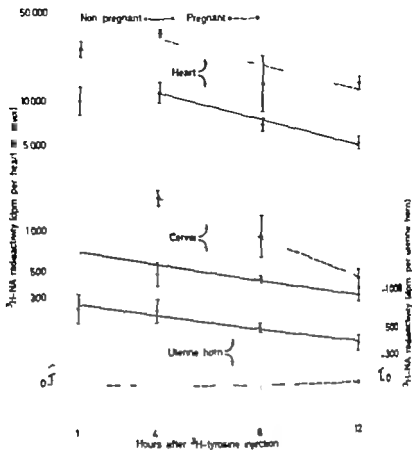


Fig. 1 The level of ^3H NA formed and the rate of its turnover in heart and uterus following a single injection of ^3H tyrosine. Maximum ^3H NA was seen within 1 h in the non-pregnant uterus, and at 4 h in the other tissues. The log ^3H NA decay was linear with time. No ^3H NA formation occurred in pregnant uterine horn. Number of determinations at each point = 4. The values for slope and half-life together with statistical analysis, are presented in Table II.

centration of the heart. The amount of ^3H NA formed from ^3H -tyrosine at 4 h after injection varied in a similar way. Thus, ^3H NA was considerably higher in the hearts of pregnant animals when calculated per whole organ, but no significant difference was found if ^3H NA was expressed in terms of specific activity (dpm/g tissue w/w). Chemical sympathectomy produced marked decreases in both total content (95%) and concentration (96%) of endogenous NA. Also the ^3H NA formation was drastically reduced by this, to only about 10% (irrespective if calculated per organ or on weight basis). ^3H NA was formed at a much lower rate in the hearts of pregnant animals compared to virgin control animals.

In hearts of both pregnant and non-pregnant animals the amount of ^3H NA increased up to 4 h, when the highest values were observed (Fig. 1). Subsequently 8 and 12 h after injection the amount of ^3H NA formed decreased. There was a linear relationship (Fig. 1) between time and log radioactivity of ^3H NA (dpm) formed per organ in the 4–12 h interval (non-pregnant, $p < 0.01$; pregnant, $p < 0.05$) and there was a significant negative slope for the two lines (non-pregnant $p < 0.001$; pregnant, $p < 0.025$). The slope (k) of the regression line, expressing the turnover rate of the ^3H NA, was virtually the same in the hearts of

Our study indicates that significant amounts of ^3H -tyrosine have been available for NA synthesis also in the interval between 1 and 4 h. The turnover and half-life estimates are based on the assumption that labelled precursor is no longer available during the period of ^3H -NA decay (Persson 1969, Weiner 1974). Therefore the estimation of the turnover started with the highest values for ^3H -NA formed. The figures obtained for turnover rate and apparent half-life in the heart of both pregnant and non-pregnant animals well within the range reported by others (cf Östman and Nybäck 1976, Kennedy and Marshall 1977).

During pregnancy the weight of the heart and its NA content was considerably increased. In view of the fact that the transmitter concentration per unit weight remained unchanged, it is probable that the phenomenon represents an increased functional demand on the heart during pregnancy. Also the ^3H -NA formation from ^3H -tyrosine was found to keep pace with target weight gain. The observations may be looked upon as examples of the preservation of a constant relationship between the terminal nerve plexus and its target tissue under physiological circumstances (Hendry 1976).

The innervation of the internal male genital organs is innervated exclusively by the autonomic nervous system, and the type of short adrenergic neurons (Sjöstrand 1965, Öwman, Sjöberg and Sjöstrand 1975, Rosengren, Öwman and Sjöberg 1975). This arrangement has made it possible to analyze pharmacological and physiological differences between these neurons and the classical sympathetic nerves, which arise in para- and prevertebral ganglia. Using a variety of approaches it has been found that the short adrenergic neurons constitute a functionally unique system of nerves with several peculiarities recently listed and discussed by Rosengren *et al.* (1975). One is a particularly slow transmitter turnover (Swedin 1971). In short adrenergic neurons represent a considerable contribution to the sympathetic innervation of the guinea-pig uterus (Thorbert *et al.* 1977). From the results of fluorescence microscopy and fluorimetric determinations of NA after various types of pharmacological blockade it has been suggested that the uterine adrenergic nerves of the guinea-pig have a low functional activity compared to the heart, possibly reflecting this marked contribution of short adrenergic neurons (Thorbert *et al.* 1978 b). The mixed population of adrenergic nerves is well reflected in the smaller difference in comparison with the heart than the usually reported difference between heart and vas deferens (Swedin 1971, Bralet, Beley and Bralet 1971). The direct measurement of ^3H -NA turnover in the present study confirmed these observations by showing a lower turnover rate in the uterine horns and cervix than in the heart. Also the oviduct of the rabbit has a mixed population of "long" and "short" adrenergic neurons (Öwman, Rosengren and Sjöberg 1966). Based on the rate of appearance of endogenous NA following synthesis inhibition with α -methyl- p -tyrosine, it was recently indicated that the NA turnover is higher in the heart than in the oviducts, supporting the concept that the adrenergic innervation of the genital tract represents a functionally different system of peripheral sympathetic nerves (Kennedy and Marshall 1977).

The adrenergic innervation in the uterine horns during the growth of the conceptus has been studied by various techniques in, particularly the guinea-pig. A total disappearance of uterine adrenergic nerves (Thorbert *et al.* 1978 b) and a parallel drastic reduction in

Uterine horn. Chemical sympathectomy by 6-OH DA reduced the content and concentration of endogenous NA by about 95%. The total amount ^3H NA formed from ^3H -tyrosine was 3 and the corresponding specific activity of ^3H NA 6% of that in organs with rich adrenergic innervation. Even more pronounced reductions were seen in late pregnancy where NA values were at or below the limit of detectability and no ^3H NA formation could be established. 4 of the animals injected with ^3H -tyrosine (at 4 and 12 h) carried fetuses out in one of the uterine horns. Thus, the formation of ^3H NA in the empty horn could not be analyzed separately. The radioactivity was below the level of detection also in these horns.

The highest value for ^3H NA formed in the uterine horns from non-pregnant animals was found at 1 h, and a decrease was observed with longer observation times (Fig. 1). The relationship between log tissue ^3H NA and time was linear ($p < 0.05$). The turnover rate was very similar to that found in the cervix of non-pregnant animals (half-life about 19 h) but slower than that in the heart ($p < 0.05$).

Discussion

Various methods have been used to estimate the NA turnover in adrenergic nerves (for reviews, see Costa 1972, Weiner 1974). One very frequently used approach involves measurement of the decrease in endogenous NA after synthesis inhibition by α -methyl- p -tyrosine. This has certain limitations, e.g. incomplete inhibition of the tyrosine hydroxylase (Widerlöv 1977) or change in neuronal activity caused by the interfered synthesis (Weiner 1977, Widerlöv 1977). Another method measures the disappearance of labelled NA injected as tracer doses (Costa 1972). In a recent study on incubated myometrial tissue slices we have shown that ^3H NA is efficiently taken up from the incubation medium, and that this uptake can be separated into 3 phases: neuronal uptake, extraneuronal uptake, and passive distribution in the extracellular space (Alm *et al.* 1979b). In that study it was shown that the neuronal uptake decreased drastically during pregnancy and that the extraneuronal accumulation was reduced to a smaller extent implying that this part of the total uptake becomes relatively more important during pregnancy. For this reason, the use of ^3H NA administration to estimate the rate of NA turnover in the pregnant myometrium has the obvious risk of determining predominantly the extraneuronal turnover of NA.

In this particular situation, the measurement of ^3H NA disappearance after ^3H -tyrosine administration is more appropriate because it measures the ^3H NA formed intraneuronally and it represents another widely used method for the estimation of catecholamine turnover (cf. Weiner 1974). After a single i.v. injection of the labelled NA precursor the initial increase in radioactive NA probably reflects the actual rate of transmitter synthesis (Schoff *et al.* 1968). After a period of 15–30 min, the tissue content of labelled NA is the net result of liberation, uptake, and metabolism of the amine together with a continuous synthesis of labelled transmitter from various tissue pools of exogenous tyrosine (Persson 1969). The possible limitations of the ^3H -tyrosine method reduces its validity in calculating the actual synthesis rate of NA (Persson and Waldeck 1970), but a general consensus seems to exist that it is reliable for comparative purposes (Weiner 1974). The rising ^3H NA activity in three of the tissue preparations studied even 4 h after ^3H -tyrosine administration, as found in the

17. CE. OSMAN, N.-O. SÖDERBERG and G. THORBERT Uptake and metabolism of H-norepinephrine by the sympathetic nerves of the guinea-pig. Effect of pregnancy *Amer J Physiol* 1977, 134, 134-138.
18. H. G. CE. OSMAN and N.-O. SÖDERBERG, Neural mechanisms in male fertility I J J. Sciatta, M. L. and J. J. Spindel (eds.), *Control of Male Fertility* Harper and Row New York 1975, p. 26-40.
19. R. R. DE OLLANGS and R. H. ... an intracervical segment. *Gynecologia* 1972, 21-26.
20. R. A. P. BILLY L. ROCHTER and J. BRALLET Evolution in the synthesis rate of catecholamines in the peripheral organs of the rat during cold exposure. *Pflügers Arch. ges. Physiol* 1976, 366, 24-28.
21. R. A. C. CARLSON, E. ROSENBERG and B. WALDECK, A method for the fluorometric determination of norepinephrine and dopamine in tissues. *Acta physiol. scand.* 1958, 44, 273-292.
22. J. A. BILLY and A. M. BRALLET Taux de renouvellement de la noradrénaline dans le canal déférent et les vésicules adrénales du rat. *Experientia (Basel)* 1971, 27, 787-788.
23. R. L. Sample neuronal models to estimate turnover rate of noradrenergic transmitters in vivo. In C. C. and E. G. G. (eds.) *Biochemistry of Simple Neuronal Models. Advances in Biochemical Pharmacology* Raven Press, New York, 1972, 2, 109-204.
24. R. L. The prenatal growth of the guinea-pig. *Amer. Rev.* 1920, 18, 349-392.
25. S. CE. OSMAN and N.-O. SÖDERBERG, Recovery of the transmitter content in uterine adrenergic nerves after pregnancy *Amer J Obstet Gynecol* 1971, 109, 997-1002.
26. A. L. ARONOW and S. M. KALMAN, Principles of drug action. Hoeber Medical Division, New York and Rev. Publ. Inc. 1969, 21-22.
27. J. A. An improved method for fluorometric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *Acta physiol. scand.* 1963, 39, 243-257.
28. J. A. Control in the development of the vertebrate sympathetic nervous system. In S. Ehrenfeld and L. J. Korn (eds.) *Essays of Neurochemistry*, Vol. 2 Raven Press, New York, 1976, p. 149-194.
29. P. De Mierstra-nucleo-placenta and the embryo. *Z. Anat. Entwickl. Gesch.* 1969, 133-135.
30. D. R. and L. M. MARSHALL, Effect of adrenergic nerve stimulation on the rabbit oviduct. Correlation with norepinephrine content and turnover rate. *Biol. Reprod.* 1977, 17, 200-211.
31. P. B. and L. AXELSON, Biochemistry of catecholamines. *Amer. Rev. Biochem.* 1971, 40, 445-500.
32. L. and H. NYLÉN, Adaptive changes in central and peripheral noradrenergic neurons in rat following chronic exercise. *Neuroscience* 1976, 1, 41-47.
33. C. E. ROSENBERG and N.-O. SÖDERBERG, Origin of the adrenergic innervation to the female genital tract of the rabbit. *Life Sci.* 1966, 5, 1395-1396.
34. C. E. ROSENBERG and N.-O. SÖDERBERG, Short adrenergic neurones—a peripheral neuroendocrine system. In M. Fajers and C. Tanaka (eds.) *Adrenal Fluorescence Fluorescence* Igaku-Shoin, Tokyo 1974, p. 47-64.
35. C. E. P. ALM, E. ROSENBERG, N.-O. SÖDERBERG and G. THORBERT Variations in the level of uterine norepinephrine during pregnancy in the guinea-pig. *Amer J Obstet Gynecol* 1975, 122, 961-964.
36. L. T. Catecholamine turnover in central nervous system. *Läkemedelsöfverlägen, Göteborg, Sweden* 1970.
37. T. Control and peripheral catecholamine turnover studied by means of ³H DOPA and H-tyrosine. *J. Pharmacol. (Kob.)* 1969, 27, 397-409.
38. T. and B. WALDECK, The use of ³H-DOPA for studying cerebral catecholamine metabolism. *J. Pharmacol. (Kob.)* 1968, 26, 363-372.
39. T. and B. WALDECK, Some problems encountered in attempting to estimate catecholamine turnover by labelled tyrosine. *J. Pharm. Pharmacol.* 1970, 22, 473-478.
40. L. L. The application of the principles of statistical analysis to the biological assay of hormones. *Endocrinology* 1946, 39, 161-176.
41. P. ALM, C. E. ROSENBERG, N.-O. SÖDERBERG and G. THORBERT Ultrastructural evidence for adrenergic innervation in the guinea-pig uterus during pregnancy. *Cell Tiss. Res.* 1979, 199, 1-10.
42. G. C. V. K. W. and L. J. K. W. The rate of norepinephrine synthesis measured in vivo during the prenatal influence of adrenergic nerve impulse activity. *J. Pharmacol. exp. Ther.* 1963, 159, 274-282.
43. D. The adrenergic innervation of the male deferens and accessory male genital glands. *Acta Physiol. Scand.* 1963, 63, Suppl. 257.

the NA content have been reported (Owman *et al* 1975). The axonal uptake mechanism for NA deteriorates (Alm *et al* 1979 b) and the tyrosine hydroxylase activity, the rate limiting step in the NA synthesis, becomes reduced below the level of detectability towards the end of pregnancy (Alm *et al* 1979 a). The appearance of the nerve plexus after silver impregnation (Gårdmark *et al* 1971) and in histofluorescence studies (Thorbert *et al* 1978 b) has suggested that these changes are due to degenerative phenomena. This has been verified in electron microscopic studies (Sporrøng *et al* 1979). The presently reported finding of no measurable ^3H NA in tissues from fetus-containing uterine horns after ^3H -tyrosine administration is directly in line with this. A further support was obtained in the experiment with 6-OH DA where the chemical sympathectomy resulted in a reduction of the ^3H -formation of the same magnitude as that found in late pregnancy.

Although the NA content in the cervix, like that in the uterus, is reduced to very low levels at the end of pregnancy (Owman *et al* 1975), the adrenergic nerve plexus appears to be functionally intact in this part of the uterus (Thorbert *et al* 1978 b). The ^3H -NA formation capacity at 4 h was found to be clearly higher in pregnant than in non-pregnant animals, suggesting an increased *in vivo* synthesis of transmitter. In accordance with this, the k_{app} value (reflecting the turnover rate) was significantly higher in the pregnant animals, and as a consequence of this, the apparent half life shorter in the cervix of pregnant animals (4 h) than in the non-pregnant controls (9 h). One factor that to a high degree influences the turnover rate is the impulse traffic in the neurons (Sedvall *et al* 1968; Costa 1972; West 1974). In the light of this it is reasonable to assume that the cervix in late pregnancy may be under an increased adrenergic influence. It has been shown that neuronal hyperactivity is related to decreased transmitter levels (Beley *et al* 1976). Thus a high neuronal activity associated with a high NA turnover may be one reason for the low NA in the cervix during late pregnancy. There are, in addition, other possible explanations to this reduction, e.g. increased metabolic breakdown of transmitter or impairment of the intraneuronal storage function leading to a decreased transmitter retention.

Nerve-mediated contractility of the cervix has been suggested as an important factor for the maintenance of human pregnancy (Bayer 1961). However, this idea has not been further pursued, probably due to the lack of knowledge about the anatomical and functional peculiarities of the adrenergic innervation in this region. The present indirect evidence for increased adrenergic impulse traffic in the cervix in late pregnancy gives new support to the idea that neurogenic mechanisms may be of importance in the maintenance of pregnancy, i.e. a sphincter mechanism.

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References

- ALM, P., A. BJÖRKLUND, CH. OWMAN and E. THORBERT. Tyrosine hydroxylase and DOPA decarboxylase activities in the guinea-pig uterus. Further evidence for functional adrenergic innervation in association with pregnancy. *Neuroscience* 1 (in press, 1979).

Effects of indomethacin on regional blood flow in conscious rabbits—a microsphere study

By

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Abstract

L. A. Effects of indomethacin on regional blood flow in conscious rabbits—a microsphere study. Acta physiol. scand. 1979 105 437-442.

In order to reveal the importance of prostaglandins in the control of regional blood flow 20 mg/kg indomethacin was given i.v. in conscious resting rabbits. Regional blood flow determinations were made before and 20 min after the injection using the labelled microsphere technique. The blood flow in skeletal wall was reduced by 0.75 ± 0.17 g min⁻¹ g⁻¹ from a level of 1.64 ± 0.24 g min⁻¹ g⁻¹ to the corresponding figures were 0.44 ± 0.12 and 1.26 ± 0.17 and in the brain 0.29 ± 0.10 and 1.24 ± 0.17 g min⁻¹ g⁻¹. The blood flow in the liver via the hepatic artery increased by 0.20 ± 0.02 g min⁻¹ g⁻¹ from a level of 0.17 ± 0.02 g min⁻¹ g⁻¹. In the retina there was a reduction in blood flow by 2.75 ± 1.03 mg min⁻¹ from a resting level of 11.1 ± 2.3 mg min⁻¹. In a number of other tissues investigated there are no significant effects of the drug. The results suggest that under resting conditions prostaglandins play a role in control of blood flow in the gastrointestinal tract, the brain and the retina—tissues which are likely to show active under such conditions.

Key words: indomethacin, prostaglandins, anaesthetized rabbit, regional blood flow

It is well established that prostaglandins have strong direct vasomotor effects, are involved in inflammatory reactions and that ischemia tends to cause release of vasodilating prostaglandins at least in skeletal muscle and the kidneys. But prostaglandins also seem to enhance the effects of autonomic nervous stimulation (Samuelsson and Wernström 1971 and Wernström 1974). Prostaglandins therefore can be expected to be involved in the control of regional blood flow in many tissues—especially under conditions of distress with high activity.

The physiological role of prostaglandins under normal conditions is not well-defined. There is evidence suggesting involvement of prostaglandins in vasomotor control has been found for the kidneys (Owen *et al.* 1975, Herbaczynska-Cedro and Vane 1973, Longro *et al.* 1977), Alosio Bandow and Rowe 1974, Bellin and Bhattacharya 1977), brain (Pickard *et al.* 1977), retina (Pournaras, Tsacopoulos and Chaplin 1978) and stomach (Gellera *et al.* 1977). So far most studies on prostaglandin effects were per-

- SWEDEN, G., Studies on neurotransmission mechanisms in the rat and guinea-pig vas deferens. *Arch. phys. scand.* 1971 *Suppl.* 369 1-34.
- THORBERT G, P ALM, CH OWMAN and N.-O SJÖBERG, Regional distribution of adrenergic nerve in guinea-pig uterus. *Amer. J. Physiol.* 1977 *233* C25-C34.
- THORBERT G, P ALM, CH OWMAN, E. ROSENBERG and N.-O SJÖBERG, Differential effect on the sympathetic transmitter level in uterus and other organs by drugs interfering with adrenergic nerve function. *Acta physiol. scand.* 1978 *a* 104 203-212.
- THORBERT G, P ALM, CH OWMAN, N.-O SJÖBERG and H SPORRÖN, Regional changes in structural and functional integrity of myometrial adrenergic nerves in pregnant guinea-pig, and their relationship to localization of the conceptus. *Acta physiol. scand.* 1978 *b* 103 120-131.
- WEINER, N. A critical assessment of methods for the determination of monoamine synthesis turnover *in vivo*. In E. Usdin (ed.) *Neuropsychopharmacology of Monoamines and their Regulatory Enzymes*. Raven Press, New York 1974 *12* 143-159.
- WIDERLÖW E., Brain catecholamines and amphetamine antagonism after acute and chronic α -methyl tyrosine administration. *Acta Ule. Upsalienensis* 1977 *283* 1-33.

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With indomethacin, prostaglandins, conscious rabbit, regional blood flow

It is well established that prostaglandins have strong direct vasomotor effects, are involved in inflammatory reactions and that ischemia tends to cause release of vasodilating substances at least in skeletal muscle and the kidneys. But prostaglandins also seem to modulate the effects of autonomic nervous stimulation (Sawchenko and Wernholm 1971 and Wernholm 1974). Prostaglandins therefore can be expected to be involved in control of regional blood flow in many tissues—especially under conditions of distress.

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TABLE 1 Effects of 20 mg/kg b.wt. indomethacin on regional blood flow in 15 rabbits, $M \pm SE$

Tissue	Normal flow $g \min^{-1} g^{-1}$	Flow after indomethacin $g \min^{-1} g^{-1}$	Change $g \min^{-1} g^{-1}$	
Heart muscle (left ventricle)	5.82 ± 0.74	6.07 ± 0.74	0.25 ± 0.33	ns
Diaphragm	0.88 ± 0.08	0.92 ± 0.14	0.038 ± 0.11	ns
Skeletal muscle	0.22 ± 0.03	0.20 ± 0.03	-0.021 ± 0.05	ns
Brain	1.24 ± 0.10	0.95 ± 0.10	-0.29 ± 0.10	$p = 0.01$
Choroid plexus	5.17 ± 0.39	5.41 ± 0.90	0.25 ± 0.87	ns
Adrenals	1.37 ± 0.14	1.52 ± 0.16	0.15 ± 0.09	ns
Kidney cortex	6.35 ± 0.47	6.04 ± 0.52	-0.31 ± 0.37	ns
Stomach wall	1.64 ± 0.44	0.89 ± 0.11	-0.75 ± 0.17	$p = 0.001$
Jejunum	1.26 ± 0.17	0.82 ± 0.12	-0.44 ± 0.12	$p = 0.01$
Liver (hep. artery)	0.13 ± 0.02	0.33 ± 0.03	0.20 ± 0.02	$p < 0.001$
Spleen	3.88 ± 0.42	3.89 ± 0.83	0.007 ± 0.69	ns
Gall bladder	2.20 ± 0.26	2.30 ± 0.3	0.10 ± 0.27	ns

formed in isolated preparations or in anesthetized animals. A recent exception (Nies and Wennmalm 1978)—a study in unanesthetized man—indicated vasoconstrictive effects of indomethacin in the kidneys and splanchnic area which was interpreted to indicate that blood vessels in these regions are normally under the influence of prostaglandins.

In the present study an attempt was made to reveal a physiological influence of prostaglandins on regional blood flow in resting unanesthetized rabbits. Labelled microspheres were used to determine regional blood flow in different tissues before and after administration of indomethacin. The dose chosen was such as to cause marked inhibition of prostaglandin synthesis.

Methods

Albino rabbits of both sexes and weighing 1.9–2.7 kg were employed. One day before the blood flow measurements one femoral artery was cannulated with a polyethylene tubing for blood sampling and the left heart ventricle was cannulated via the left subclavian artery using another tubing. The tubing was filled with a heparin solution to prevent clotting. The cannulation was performed with the animals under pentobarbital anaesthesia. On the next day the animal was placed in a open box, 1000 IU Heparin injected i.v. and the blood pressure was measured through the tubing placed in the femoral artery. C was taken not to upset the animal. About 1 ml of suspension of ^{51}Cr -labelled 15 μm microspheres (Company St Paul Minnesota) were injected into the left heart ventricle over 10–15 s. From the start of the injection and for the next 60 s blood was collected from the tubing in the femoral artery. The blood flow was about 1 ml/min and the blood was collected in 10 s samples. The measurement of the blood pressure was then resumed and indomethacin, 20 mg/kg b.w. was injected i.v. The indomethacin had been dissolved in a phosphate buffer pH 8.0, immediately before the injection. The volume injected was 10 ml. Sonication was used to suspend the indomethacin not dissolved. There was little effect of the injection on the blood pressure. About 20 min later a new blood flow measurement was made using ^{51}Cr -labelled 15 μm microspheres. Each injectate contained about 1.7 million microspheres. After the second injection the animal was killed by an overdose of pentobarbital sodium followed by saturated KCl.

In 5 control experiments 10 ml of the phosphate buffer was injected without indomethacin. Flow determinations were made as in the experiments with the drug.

Assay

The amount of labelled spheres present in the tissue and blood samples was determined by tritium gamma spectrometry and the regional blood flow was calculated as described elsewhere (Stjernstrom Alm and Bill 1976).

TABLE I. Effect of 20 mg/kg b. i. indomethacin on regional blood flow in the eyes of 10 rabbits, $M \pm S.E.$

	Normal blood flow mg/min	Flow after indomethacin mg/min	Change mg/min	
Cornea	15.1 \pm 2.3	12.4 \pm 2.4	-2.75 \pm 1.03	$p < 0.025$
Retina	1000 \pm 111	930 \pm 83	-66.9 \pm 64.4	ns
Iris	68.2 \pm 7.6	72.8 \pm 6.3	4.59 \pm 7.42	ns
Optic nerve	71.6 \pm 5.1	70.5 \pm 5.1	-1.10 \pm 4.0	ns

Results

Mean arterial blood pressure just before the first blood flow measurement was 85.3 ± 2.28 mmHg. 5 min after the injection of indomethacin the mean arterial pressure was 83.8 ± 1.9 mmHg. About 20 min later just before the second blood flow measurement it was 81.6 ± 1.8 mmHg.

Table I and II show that indomethacin had no or only small effects in most tissues. There were notable exceptions. In the stomach wall there was a marked reduction in the blood flow and in the liver the hepatic arterial blood flow more than doubled. The blood flow in the small intestine was also markedly reduced. The reductions in the cerebral and retinal blood flows are small but statistically significant.

Injection of phosphate buffer without indomethacin had no appreciable effect on the blood flow in any of the tissues investigated.

Discussion

These results indicate that in unanaesthetized rabbits indomethacin causes a marked redistribution of the blood flow in the liver and gastrointestinal tract and also reduced the blood flow in the brain moderately. But in most tissues the effects on the blood flow—if any—were small.

Indomethacin concentrations of less than 25 μ g/ml have been reported to abolish the output of prostaglandins from isolated perfused rabbit hearts (Pham-Huu-Chanh, Junstad and Svensson 1972) and 20 mg/kg body weight was reported to abolish the release of prostaglandin from the kidney (Herbachyzynska-Cedro and Vane 1973). Doses as low as 1 mg/kg b. i. were reported by Oluf Lunden and Ånggård (1978) to cause marked inhibition of prostaglandin synthesis in rabbit kidneys. The dose of indomethacin used in the present experiments, 20 mg/kg b. i. w., thus can be expected to have produced a marked reduction in prostaglandin production.

Gastrointestinal tract. Gastric mucosal erosions are side effects of nonsteroid antiinflammatory drugs. Whittle (1977) reported flow measurements in urethane anesthetized rats during saline perfusion of the stomach and observed that indomethacin caused a blood flow reduction but had no consistent effect on acid loss or the potential difference across the gastric mucosa. It is suggested that microcirculatory regulation by prostaglandins may be more important in pathophysiological conditions. A recent study by Gerkens *et al.* (1977) in dogs

under general anesthesia indicated that indomethacin reduced resting blood flow in the stomach wall but not in the small intestine. Hyperemia produced by injection of pentagastrin was counteracted in the small intestine as well as in the stomach. Acid secretion under these conditions was increased by indomethacin. The present results indicate that gastric blood flow is reduced by indomethacin even in resting unanesthetized animals and that flow in the small intestine is reduced as well.

In the experiments by Nowak and Wennmalm (1978) there was a reduction in splanchnic blood flow as determined from the elimination in the liver of indocyanine green dye. To liver blood flow thus was reduced in their experiments.

Since labelled microspheres are caught in the first capillary bed through which they pass, liver blood flow measured with microspheres is essentially hepatic artery blood flow. The rather marked increase in flow observed may be an effect of reduced prostaglandin production in the walls of the hepatic resistance vessels or it may be secondary to a reduction in blood flow through the stomach and the small intestine. The latter alternative seems more likely since it is well known that hepatic artery flow tends to increase when portal venous flow is reduced (see Bradley 1963) and at least in dogs PGA and PGE₂ tend to reduce vascular resistance in the hepatic artery and its branches (Hansson and Post 1974).

Kidney. Several studies on the effects of indomethacin in anesthetized animals (Owen 1975, Herbaczynska-Cedro and Vane 1973, Lonigro *et al.* 1973) suggested that in the kidney prostaglandins are continuously involved in the control of blood flow. A report by Smith *et al.* (1975) indicated however that in unanesthetized dogs with an undisturbed renal circulation indomethacin had practically no effect on the renal blood flow. A marked effect, reactive hyperemia after short lasting ischemia was observed indicating that prostaglandins probably play a role under certain conditions such as anesthesia and hemorrhage. But the possibility of species differences and regional effects in the kidney also has to be considered. In a recent study by Bellin and Bhattacharya (1977) in unanesthetized rabbits indomethacin tended to reduce renal blood flow but the effect was marked only in the inner cortex. In the experiments by Nowak and Wennmalm (1978) in unanesthetized man also indicated renal vasoconstriction after indomethacin—an effect that was overcome by infusion of PGE₂. In the present experiments, the cortex was not divided into different parts, the samples containing mainly middle cortex. As in Bellin and Bhattacharya's study there was no statistically significant effect in this region.

The eye. Prostaglandins injected into the anterior chamber have very marked effects on the blood vessels of the anterior uvea, causing vasodilatation and capillary damage even at low concentrations (Eakins 1977). The lack of effect of indomethacin in the anterior chamber indicates that there is no significant liberation of vasoactive prostaglandins in this tissue under normal conditions. In the retina there was a slight reduction in flow suggesting that significant amounts of prostaglandins may be released in this tissue. A small effect of indomethacin on the calibre of retinal vessels has recently been observed also by Pournazeri, Tsacopoulos and Chapuis (1978). These results support the hypothesis of Bilo (1977) that the physiological role of a prostaglandin accumulating mechanism present in the ciliary processes is to remove prostaglandins diffusing forward from the retina and threatening the anterior uvea.

The results. In a previous study with close arterial infusion of indomethacin in anesthetized dogs Pickard and Mackenzie (1973) observed a 38% reduction in cerebral blood flow to a baseline value of 0.57 ml min⁻¹ g⁻¹ tissue but there was no effect on the oxygen consumption. The response of the cerebral vessels to hypercapnia was markedly reduced. In the present experiments the reduction in blood flow was rather small and variable but the results support the hypothesis that prostaglandins may be involved in the control of cerebral blood flow.

Work. Recent reports suggest that indomethacin affects resting blood flow in skeletal muscle very little but that reactive hyperemia and hypertemia during work is markedly reduced after indomethacin pretreatment (Kilborn and Wennumalm 1976, Messina, Weiner and Kaley 1977). Indomethacin has been reported to reduce coronary blood flow in isolated preparations from guinea pigs (Schröber, Krebs and Nookhwun 1976) but to have no significant effect on coronary blood flow isolated rabbit hearts (Needleman *et al.* 1975). Effects of hypoxia were reduced in dogs under general anesthesia (Alonso Bando and Mac 1974) but no such effects could be demonstrated in anoxic rabbit hearts (Block *et al.* 1974). In the present study indomethacin affected neither skeletal muscle blood flow nor coronary perfusion in a detectable way.

One can speculate on the basis of previous and the present results that prostaglandin release is related to the activity of the tissues and tends to increase in anesthesia and due to hypoxia. In the present study the lack of effect of indomethacin on most tissues may have been due to the resting state of the animals. The effects on the gastrointestinal tract may have been a relatively high activity in this region as can be expected under the conditions of the study.

Indomethacin may affect vascular tone in other ways than by inhibiting prostaglandin release (Northover 1977). Therefore more definite conclusions concerning the physiological role of prostaglandins in the control of vascular tone will have to be based on further studies with various other agents inhibiting prostaglandin synthesis or action.

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References

- Alonso Bando, G. T. and G. G. Rowe, Indomethacin and the prostaglandin hypothesis of coronary blood flow regulation. *J. Physiol. (Lond.)* 1974, 241, 299-308.
- Block, A. L. and J. B. BARTCHART, The effect of prostaglandin synthesis inhibitors on renal blood flow in conscious rabbits. *J. Pharmacol. (Lond.)* 1977, 269, 395-403.
- Block, A. L. and M. C. WALLENTIN, Transport of prostaglandins across the blood-brain and blood-nerve barriers and the physiological significance of their absorptive transport processes. *Exp. Eye Res.* 1977, 25, Suppl. 279-283.
- Block, A. L., H. FINNBERG, K. HERRICKSBERG-CHEN and J. R. V. M., Anoxia-induced release of prostaglandins in rabbit isolated hearts. *Circulat. Res.* 1975, 36, 34-42.
- Block, A. L., The hepatic circulation. In: *Handbook of Physiology: Circulation*. American Physiological Society, Washington, 1963, II, 1387-1438.
- Block, A. L., Prostaglandin and non-prostaglandin mediated breakdown of the blood-aqueous barrier. *Exp. Eye Res.* 1977, 25, Suppl. 481-498.

- GERKENS, J. F. D. G. SHAND, C. FLEXNER, A. S. NIES, J. A. OATES and J. L. DATA, Effect of indomethacin and aspirin on gastric blood flow and acid secretion. *J. Pharmacol. exp. Ther.* 1977, 203, 644-652.
- HANSSON, K. M. and J. A. POST, Splanchnic vascular responses to the infusion of prostaglandin E_2 and B . *Pharmacology* 1976, 14, 166-181.
- HERBACZYNSKA-CEDRO, K. and J. R. VANE, Contribution of intrarenal generation of prostaglandins to autoregulation of renal blood flow in the dog. *Circulat. Res.* 1973, 33, 428-436.
- JUNSTAD, M. and Å. WENNMALM, Release of prostaglandin from the rabbit isolated heart following nerve stimulation or acetylcholine infusion. *Brit. J. Pharmacol.* 1974, 52, 375-379.
- KILBOM, Å. and Å. WENNMALM, Endogenous prostaglandins as local regulators of blood flow in a: effect of indomethacin on reactive and functional hyperaemia. *J. Ph. stat. (Lond.)* 1976, 257, 109-112.
- LONGRO, A. J. H. D. ITSKOVITZ, K. CROWSHAW and J. C. MCGIFF, Dependency of renal blood flow on prostaglandin synthesis in the dog. *Circulat. Res.* 1973, 3, 712-717.
- MISHRA, E. J. R. WEINER and G. KALEY, Arteriolar reactive hyperemia: modification by inhibition of prostaglandin synthesis. *Amer. J. Physiol.* 1977, 232, H571-H575.
- NEEDLEMAN, P. S. L. KEY, P. C. ISAKSON and H. S. KULKAR, Relationship between oxygenated coronary vasodilation and prostaglandin biosynthesis in the isolated rabbit heart. *Prostaglandins* 1975, 1, 13-134.
- NORTHOVER, B. J. Indomethacin—a calcium antagonist. *Gen. Pharmacol.* 1977, 8, 293-294.
- NOWAK, J. and Å. WENNMALM, Influence of indomethacin and of prostaglandin E_2 on total and regional blood flow in man. *Acta physiol. scand.* 1978, 10, 484-491.
- OLSSON, E., I. LUNDÉN and E. ÅNGSTRÖM, *In vivo* inhibition of prostaglandin synthesis in rabbit kidney: non-steroidal anti-inflammatory drugs. *Acta pharmacol. (Abh.)* 1978, 42, 179-184.
- OWEN, T. L., I. C. EHRLHART, W. J. WEIGER, J. B. SCOTT and F. J. HADDY, Effects of indomethacin on local blood flow regulation in canine heart and kidney. *Proc. Soc. exp. Biol. Med.* 1975, 149, 871-874.
- PIIAM-HUU-CHANIL, M., JUNSTAD and Å. WENNMALM, Augmented noradrenaline release following α_1 stimulation after inhibition of prostaglandin synthesis with indomethacin. *Acta physiol. scand.* 1972, 563-567.
- PICKA, D., J. H. and E. T. MACKENZIE, Inhibition of prostaglandin synthesis and the response of total cerebral circulation to carbon dioxide. *Nature New Biol.* 1973, 245, 187-188.
- POURMARAS, C. M., TRACHOPOULOS and Ph. CHAPIUS, Studies on the role of prostaglandins in the regulation of retinal blood flow. *Exp. Eye Res.* 1978, 26, 687-697.
- SAMUELSSON, B. and Å. WENNMALM, Increased nerve stimulation induced release of noradrenaline from rabbit heart after inhibition of prostaglandin synthesis. *Acta physiol. scand.* 1971, 83, 163-168.
- SCHULÖN, K., H. KREIS and C. NOOKIRWUN, Increase in the coronary vascular resistance by indomethacin in the isolated guinea pig heart preparation in the absence of changes in mechanical performance and oxygen consumption. *Europ. J. Pharmacol.* 1976, 39, 161-169.
- STERENKJANTZ, J. A. ALM and A. BILL, Effects of intracranial oculomotor nerve stimulation on ocular blood flow in rabbits. Modification by indomethacin. *Exp. Eye Res.* 1976, 23, 461-469.
- SWAIN, J. A., G. R. HAYNDRICKS, D. H. BOETTCHER and S. F. VATHER, Prostaglandin control of splanchnic circulation in the unanesthetized dog and baboon. *Amer. J. Physiol.* 1975, 229, 826-830.
- WHITTLE, B. J. R., Mechanisms underlying gastric mucosal damage induced by indomethacin and histamine and the actions of prostaglandins. *Brit. J. Pharmacol.* 1977, 60, 455-460.

Effects of 4-aminopyridine on the excitation-contraction coupling in frog and rat skeletal muscle

By

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Abstract

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The effects of 4-aminopyridine (4-AP) were studied on isolated single muscle fibres of the frog and toe adductor of the rat. In both muscle preparations, 4-AP potentiated the twitch amplitude almost independently of the tetanic response. There was an increase of the time to peak tension and, in frog muscle, a marked time to half relaxation. 4-AP produced no change of the resting membrane potential. The rate of rise and, hence, the total duration of the action potential were markedly prolonged. 4-AP did not alter membrane properties by itself nor did it affect the contracture induced by caffeine. The mechanical threshold was increased by increasing the contracture response to various degrees of depolarization by potassium. In the rat, the threshold was not affected by 4-AP. Twitch potentiation by 4-AP was independent of the extracellular Ca^{2+} concentration. It is concluded that 4-AP potentiates the twitch response by increasing the release of Ca^{2+} from the sarcoplasmic reticulum into the myofibrillar space by prolongation of the action potential. In addition, there is a direct inhibitory action of 4-AP on the calcium re-uptake by the sarcoplasmic reticulum in frog muscle.

Key words: 4-aminopyridine, skeletal muscle, single muscle fibre, excitation-contraction coupling, contracture, action potential, membrane potential.

Recently interest has been focussed on the muscular effects of 4-aminopyridine (4-AP), a agent which has been shown to counteract the paralysis caused by curare (Paskov *et al.* 1977). The action of 4-AP on neuromuscular junction has been studied and there is evidence that the agent greatly potentiates transmitter release possibly by increasing the level of Ca^{2+} at the nerve terminal (Molgo *et al.* 1975, 1977; Lundin and Thøgersen 1977). However, it is known as to whether or not 4-AP also affects the contractile performance by acting directly on the excitation-contraction coupling. This latter aspect has been elucidated in the present study. To this end the effects of 4-AP on the time course of twitch relaxation have been analyzed on single muscle fibres of the frog and on curarized toe adductor of the rat. Evidence will be presented to show that the twitch potentiation produced by 4-AP may at least partly be accounted for by prolongation of the membrane action potential.

Methods

Preparation

Frog muscle Single muscle fibres were isolated from the ventral head of the semitendinosus muscle *Rana temporaria*. For mounting the fibres, a link of stainless steel wire (thickness, 0.1 mm) is attached each tendon as described previously (Edman and Klesall 1971).

Rat toe muscle Thin toe muscles were dissected from the front legs of Sprague-Dawley rats (150–200 g). A small loop of silk thread was tied to the tendon at each end of the muscle.

Isolation chamber

The preparations (frog single fibres or rat whole muscles) were mounted horizontally in a Perspex dish between a tension transducer (see below) and an adjustable stainless steel hook, the position of which can be set by means of a micrometer screw. The chamber was 6 mm wide, 5 mm deep and contained 12 ml solution. The solution was changed by introducing fresh solution at the transducer end of the dish and it was removed by suction drain at the other end. A flush time of about 3 s is used when the previous solution was added for production of contractions. This caused an almost complete (95%) change of the bathing solution (Andersson and Edman 1974 b).

Temperature control

During an experiment the temperature was controlled by a Colson Ultra-thermostat which circulated ethylene glycol-water mixture through jackets surrounding the chamber and the containers of solution. The solution containers were connected with the chamber through polyethylene tubes (approximately 10 cm long) and a stopcock at the chamber inlet.

In studies on single muscle fibres, the bath temperature varied between 1 and 3°C and in studies on rat toe muscles between 22 and 23°C from expt. to expt. The temperature was maintained constant within $\pm 0.1^\circ\text{C}$ throughout any particular expt., even during exchange of solution.

Tension recording

Tension was recorded by means of an RCA 5734 mechano-electric transducer. The signals were displayed on a Tektronix 40 A oscilloscope and were recorded simultaneously on paper by means of an Ektaschönder ink-jet recorder.

Electrical stimulation

The single muscle fibre or the rat toe muscle was stimulated by means of a multielectrode assembly described previously (Edman and Klesall 1971). Pulses of 0.5 ms duration were used and the stimulus strength was adjusted to be supramaximal for each electrode pair. For tetanic stimulation, 1 s train pulses with a frequency of 16–20 Hz was used for the single muscle fibre. To obtain a fused tetanus in the toe muscle, 1 s train of pulses with a frequency of 100 Hz was used.

The single muscle fibre or the rat toe muscle was mounted for at least 1 h before the expt. and tetanized at 10 min intervals during this time. During the actual expt. the preparation was stimulated every 2 min (if not otherwise stated) to record either twitch or tetanic response.

Solutions

Frog muscle fibre experiments The solutions used had the following composition (mM):

Ordinary Ringer solution: NaCl 115.5, KCl 4.0, CaCl_2 1.8, Na-phosphate buffer 2.0, pH 7.0.
Calcium-free Ringer: NaCl 116.3, KCl 2.0, EDTA 1.0, Na-phosphate buffer 2.0, pH 7.0.
Lanthanum containing Ringer: NaCl 117.2, KCl 2.0, La 0.05, Tris-(hydroxymethyl)-aminomethane 2.0. The pH was adjusted to 7.0 by addition of H_2SO_4 to a final concentration of 0.94 mM.
For potassium contractions, solutions of the following composition were used:

Solution (mM)	KCl	K ₂ CH ₃ SO	Na-CH ₃ SO	NaCl	CaCl ₂
10 K Ringer	10	—	96.90	10.60	1.80
20 K Ringer	—	20	88.99	8.51	1.80
40 K Ringer	—	40	75.05	2.43	1.80
80 K Ringer	—	80	37.80	—	1.30
117.5 K Ringer	—	117.5	0.77	—	1.03



Fig. 1. Effects of 4-AP on isometric twitch (A) and tetanus (B) in frog single muscle fibre. 1. Control, frog Ringer solution. 2. In the presence of 3 mM 4-AP. Stimulus markers below base line.

Ringer solutions contained 2 mM Tris-(hydroxymethyl)- α -aminoethane. The pH was adjusted to 7.4 by addition of H_2SO_4 to a final concentration of 0.94 mM.

For *in vitro* muscle experiments, Tyrode solution (mM): NaCl 134.0, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 1.0, $MgCl_2$ 0.21, K_2HPO_4 0.9, glucose 3.3, 0.5 mg/ml of d-tubocurarine. This solution was aerated by bubbling of 95% O_2 and 5% CO_2 .

Both 4-aminopyridine (4-AP), caffeine and electrogenic are dissolved in Ringer and Tyrode solutions, respectively.

4-AP concentrations in frog muscle fibres

Four solutions of the following concentrations were used (mM): 1.0, 1.5, 2.0, 3.0 and 5.0. The solutions were loaded from pre-cooled container until plateau contracture tension was achieved. This occurred at approximately 15 s. Relaxation was produced by re-introduction of the normal Ringer solution. The interval between two successive potassium or caffeine contractures was 30–25 min.

Recording of membrane potential

Fluorinated glass capillary electrodes (about 20 M Ω) filled with 2.5 M KCl were used for intracellular recordings of membrane potentials. The reference electrode was an Ag-AgCl electrode connected to the tip through an agar-KCl bridge. The microelectrode and the reference electrode were connected via a galvanometer provided with capacitance neutralization to a Tektronix 502A oscilloscope. The signals were photographed on 35 mm film. The amplified signals were also used to modulate an audio frequency oscillator. Successful penetration was indicated by an abrupt change in frequency. When action potentials were recorded the fibre was stimulated only at one locus, approximately 5 mm from the tip of the microelectrode.

Results

1. Frog muscle fibres

Twitch and tetanus responses 4-aminopyridine (4-AP) was tested on isolated muscle fibres at concentrations ranging between 0.02 and 3.0 mM. In a concentration of 1 mM or higher 4-AP potentiated the twitch response without significantly affecting the tetanus response. Maximal twitch potentiation was obtained by 3 mM 4-AP (Fig. 1). The magnitude of the twitch potentiation was inversely related to the twitch/tetanus ratio recorded in the control Ringer solution. At full effect of 4-AP a peak twitch force of more than 90% of the tetanic tension was generally attained.

As illustrated in Fig. 1 4-AP caused no significant change in the initial rate of rise of tension. The increase in twitch amplitude was associated with an increase of the time to peak tension and a prolongation of the relaxation phase. With 3 mM 4-AP the half time to peak tension and the half time for relaxation increased by 95% and 74% of the control values, respectively (Table 1).

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Preparation

Frog muscle Single muscle fibres were isolated from the ventral head of the semitendinosus muscle *Rana temporaria*. For mounting the fibres, a 10 μ k of stainless steel wire (thickness 0.1 mm) is attached each tendon as described previously (Edman and Klemm 1971).

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Temperature control

During an experiment the temperature was controlled by a Colson Ultra-thermostat which circulated ethylene glycol-water mixture through jackets surrounding the chamber and the containers of solution. The solution containers were connected with the chamber through polyethylene tubes (approximately 10 cm long) and a stopcock at the chamber inlet.

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The single muscle fibre or the rat toe muscle was mounted for at least 1 h before the expt. and tetanized at 10 min intervals during this time. During the actual expt. the preparation was stimulated every 2 min (if not otherwise stated) to record either twitch or tetanus responses.

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3. Ringer solution contained 3 mM Tris-(hydroxymethyl)-aminomethane. The pH was adjusted to 8 by addition of H_2SO_4 to final concentration of 0.94 mM.

For the muscle experiments Tyrode solution (mM): NaCl 154.0, KCl 5.6, $NaHCO_3$ 3.6, $CaCl_2$ 1.0, $MgCl_2$ 0.21, Na_2HPO_4 0.9, glucose 5.5, 0.3 mg/ml of *D*-tubocurarine. This solution is aerated by a flow of 95% O_2 and 5% CO_2 .

4-aminopyridine (4-AP), caffeine and dantrolene were dissolved in Ringer and Tyrode solutions, respectively.

Electrodes in frog muscle fibres

Four solutions of the following concentrations are used (mM): 1.0, 1.5, 2.0, 3.0 and 5.0. The solutions are heated from pre-cooled container until plateau contracture appears as described. This occurred for approximately 15 s. Relaxation was produced by re-introduction of the normal Ringer solution. The interval between two successive contractions or caffeine contractions was 20–25 min.

Recording of membrane potential

Increased glass capillary electrodes (about 20 M Ω) filled with 2.5 M KCl were used for intracellular recordings of membrane potentials. The reference electrode was an Ag-AgCl electrode connected to the cell through an agar-Ringer bridge. The microelectrode and the reference electrode are connected via a detector provided with capacitance neutralization to Tektronix 502A oscilloscope. The signals are photographed on 25 mm film. The displayed signals were also used to modulate an audio frequency oscillator. Successful impalement was indicated by an abrupt change in frequency. When action potentials in the fibre was stimulated only at one focus, approximately 5 mm from the tip of the microelectrode.

Results

1. Frog muscle fibres

Fast and tetanus responses 4-aminopyridine (4-AP) was tested on isolated muscle fibres at concentrations ranging between 0.02 and 3.0 mM. In a concentration of 1 mM or higher 4-AP potentiated the twitch response without significantly affecting the tetanus response. Tetanic twitch potentiation was obtained by 3 mM 4-AP (Fig. 1). The magnitude of the twitch potentiation was inversely related to the twitch/tetanus ratio recorded in the control solution. At full effect of 4-AP a peak twitch force of more than 90% of the tetanic tension was generally attained.

As illustrated in Fig. 1 4-AP caused no significant change in the initial rate of rise of tension. The increase in twitch amplitude was associated with an increase of the time to peak tension and a prolongation of the relaxation phase. With 3 mM 4-AP the half time to peak tension and the half time for relaxation increased by 95% and 74% of the control values, respectively (Table I).

TABLE 1 Effects of 4-AP on twitch parameters in frog single muscle fibres. Each value represents the mean of 5-10 twitch responses.

Exp. no.	Normal Ringer		3 mM 4-AP in Ringer		T_A/T_C	R_A/R_C
	Half time to peak tension (ms) T_D	Half time for relaxation (ms) R_D	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
1	85	130	14	210	1.67	1.62
2	86	144	180	63	2.09	1.10
3	85	165	177	785	0.8	1.73
4	76	17	148	30	1.93	1.34
5	69	84	136	184	1.97	2.19
Grand mean	80	139	157	234	1.95	1.74

The effect produced by 4-AP was only partially reversible. As illustrated in Fig. 4, repeated washing of the fibre in normal Ringer lowered the amplitude of the potentiated twitch slightly. When 3 mM 4-AP was re-introduced the twitch amplitude was again increased.

To test if the extracellular calcium plays any role in the twitch potentiation produced by 4-AP, calcium was removed from the extracellular medium by repeated washing of the fibre with a calcium free Ringer solution containing 1 mM EDTA. Fig. 3 b shows, in confirmation of previous results (Edman and Grieve 1961, 1964; Jenden and Reger 1963; O'Connell 1963), that removal of calcium from the extracellular medium made the fibre insensitive to 4-AP. Addition of lanthanum to the bathing solution in a concentration (0.05 mM) sufficient to normalize the resting membrane potential in the absence of calcium (Anderson and Edman 1974a) restored the twitch response (Fig. 3 c). 4-AP (3 mM) added to the calcium-lanthanum-containing medium caused a potentiation of the twitch in much the same way as in normal Ringer (Fig. 3 d).

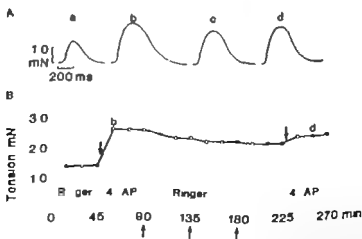


Fig. 2. Reversibility of the effects produced by 4-AP on the twitch response of frog muscle fibre. Traces in A refer to times indicated in B. Arrows above curve in B indicate addition of 3 mM 4-AP. Arrows below curve indicate washings with Ringer solution. Note that twitch potentiation is only partially reversible.

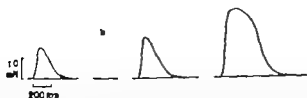


Fig. 3 Effects of 4-AP on twitch response of frog muscle fibres after removal of calcium from the extracellular solution. (a) Normal Ringer. (b) Approximately 5 min after removal of calcium (1 mM EDTA used). No twitch response. (c) After replacing calcium with 0.05 mM lanthanum. (d) Effects of 3 mM 4-AP. Calcium free Ringer containing 0.05 mM lanthanum.

Dantrolene has a powerful depressant effect on the isometric twitch. In relatively low concentrations, it greatly suppresses the twitch response of single muscle fibres (Ellis and Moxart 1972, Balmain and Desmedt 1974). The effect of dantrolene on 4-AP-treated fibres is similar. As illustrated in Fig. 4, dantrolene reduced the 4-AP potentiated twitch.

Ammonium and caffeine induced contractures Experiments were performed to find out if 4-AP affected the mechanical threshold, i.e. the membrane potential at which contractile tension is initiated. For this purpose contractures were produced by immersing the single fibre in an isosmotic solution containing potassium (constant $[K]$, $[Cl]$ product) in concentrations varying between 10 and 117.5 mM. Peak contracture tension was plotted against $\log [K]$. Fig. 5 summarizes the results obtained in three experiments, in which the effect of mM 4-AP was tested. There was a very steep rise of the contracture response in the range 10–20 mM potassium. As can be seen, 4-AP did not cause any significant change of the response to potassium depolarization.

Similarly the effect of 4-AP on caffeine induced contractures was studied. The peak tension developed was plotted against \log caffeine concentration. The S-shaped curve along contracture tension and caffeine concentration also remained unaltered in the presence of 3 mM 4-AP (Fig. 6).

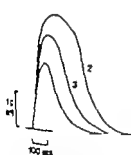


Fig. 4

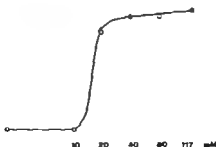


Fig. 5

Fig. 4 Effects of dantrolene on the twitch response of frog muscle fibres in the presence of 3 mM 4-AP. Normal Ringer. 2 In the presence of 4-AP. 3. In the presence of 4-AP and 9 μ M dantrolene.

Fig. 5 Relation between peak contracture tension and extracellular potassium concentration in the presence of 4-AP. Frog muscle fibres. \circ Control, no 4-AP. Δ In the presence of 3 mM 4-AP. Each point represents mean value of three different experiments.

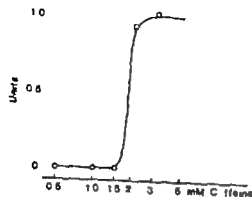


Fig. 6

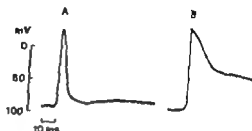


Fig. 7

Fig. 6. Relation between peak contracture tension and caffeine concentration in the presence and absence of 4-AP. Frog muscle fibres. \circ Control, no 4-AP; Δ : In the presence of 3 mM 4-AP. Each point represents mean value of three different experiments.

Fig. 7. Effects of 4-AP on intracellularly recorded action potential of frog single muscle fibres. A, no Ringer; B, in the presence of 3 mM 4-AP. The records in A and B are from different fibres.

Resting and action potentials 4-AP in a bath concentration of 3 mM did not affect resting membrane potential over at least 2 hours as tested in 13 fibres of altogether 61 bundles (Table II). The effects of 4-AP on membrane action potential were studied after twitch potentiation had developed. There were no significant changes of the maximum rate of rise and overshoot of the action potential. The maximum rate of fall and the duration of the action potential, on the other hand, were both markedly prolonged (Fig. 7 and Table II).

B. Rat toe muscle

Experiments were performed to study the effects of 4-AP on twitch and tetanic response of mammalian skeletal muscle. Curarized toe muscles of the rat were stimulated at 22 V to produce a single twitch or a 1 s fused tetanus at 2 min intervals. 4-AP in a concentration >0.1 mM caused potentiation of the isometric twitch (Fig. 8). The peak twitch amplitude was increased by approximately 1/3 of the control in response to 0.1 mM 4-AP. Similar to the situation in frog skeletal muscle 4-AP prolonged the time to peak twitch tension. However

TABLE II Effects on resting and action potentials of 3 mM 4-AP added to normal Ringer (Mean \pm S.E.) Frog muscle fibre bundles exposed to 4-AP for 15–20 min before recording the resting and action potentials. Number of fibres for each measurement given within brackets. Student's *t*-test. +++ $P < 0.001$.

	Resting potential mV	Action potential			
		Overshoot, mV	Maximum rate of rise, V/s	Maximum rate of decay V/s	Duration at -25 mV level, ms
Control	84.1 ± 2.3 (13)	33.7 ± 2.2 (10)	56.0 ± 3.5 (10)	26.4 ± 1.8 (10) +++	5.2 ± 0.2 (10) +++
4-AP (3 mM)	87.6 ± 1.8 (13)	25.5 ± 3.3 (7)	55.9 ± 4.6 (7)	4.3 ± 0.4 (7)	16.2 ± 1.6 (7)

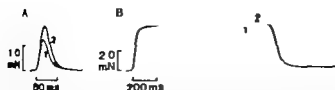


Fig. 1. Effects of 4-AP on isometric twitch (A) and tetanus (B) of a curved toe muscle of the rat. 1. Control traces. 2. In the presence of 0.1 mM 4-AP. Note different time and tension scales.

time to 50% relaxation in the rat toe muscle was not significantly affected (Table III). There was no clear effect of 4-AP on the amplitude of the fused tetanus when care was taken to stimulate the muscle supra-maximally. When submaximal stimulation strengths are used, 4-AP in concentrations >0.1 mM reduced the tetanic force. This suggests that 4-AP raised the electrical threshold thereby reducing the number of fibres activated under these conditions.

Discussion

Previous experiments (Lundh *et al.* 1977) performed on the rat extensor digitorum longus muscle suggest that 4-aminopyridine (4-AP) potentiates muscle contraction by facilitating transmitter release at the neuromuscular junction and the drug has been shown to be a partial antagonist of the neuromuscular blocking agent botulinum toxin (Lundh *et al.* 1977, Lundh and Thesleff 1977). The present results on frog single muscle fibres and on curved rat toe muscles demonstrate that 4-AP causes twitch potentiation by a more direct action on the excitation-contraction coupling. In order to produce this effect, however, considerably higher concentrations were required than those needed to affect the transmitter release (Lundh 1978).

The twitch potentiation by 4-AP is characterized by an increased peak amplitude and an increase of the half time to peak tension and of the half time for relaxation. These changes are associated with a broadening of the action potential. As previously demonstrated on single muscle fibres (Edman *et al.* 1966) and in experiments on whole muscle (Sandow and Taylor 1964, Sandow Taylor and Preiser 1965 Taylor *et al.* 1972) the duration of the released activity appears to be quantitatively related to the action potential duration (at the 25 mV level). This has been suggested to mean, that the action potential governs

Table III. Effects of 4-AP (0.1 mM) on the twitch parameters of curved toe muscles of rat. Each value represents the mean of 3-4 responses.

Expt. no.	Normal Ringer		3 mM 4-AP in Ringer		T_A/T	R_A/R_0
	Half time to peak tension (ms) T_0	Half time for relaxation (ms) R_0	Half time to peak tension (ms) T_A	Half time for relaxation (ms) R_A		
1	14	30	18	29	1.36	1.03
2	12	17	14	17	1.17	1.00
3	15	23	16	20	1.07	0.87
4	14	23	16	22	1.20	0.97

the time during which activator Ca^{2+} is released into the myofibrillar space (Sadow *et al.* 1964, Edman *et al.* 1966, Taylor *et al.* 1972). The longer duration of the action potential would cause a higher peak concentration of Ca^{2+} at the contractile sites, and this in turn would require a longer time for the elimination of Ca^{2+} from the myofibrils. If the concentration of the activator Ca^{2+} were large enough to fully activate the contractile system, a further increase of the Ca^{2+} concentration would merely cause a prolongation of the mechanical activity. This would be reflected in the isometric twitch response as an increased peak amplitude and a later attainment of peak tension, whereas the initial rate of rise of the twitch tension would remain unaffected. The effects produced by 4-AP are consistent with the idea that twitch potentiation is due to the prolongation of the action potential according to the above mechanism. However the results clearly show that 4-AP also prolongs the relaxation time in frog muscle suggesting that 4-AP exerts a more direct inhibitory action on the resequestration of calcium in this tissue.

It is of interest to note in this connection that 4-AP does not affect the mechanical threshold, *i.e.* the potential level at which contraction is initiated in response to membrane depolarization. This is inferred from the finding that 4-AP does not shift the curve relating potential concentration and contracture response (Fig. 5).

Caffeine and 4-AP affect the time course of the isometric twitch in a similar way. Previously it was considered that the effect of caffeine is due to an inhibitory action on the calcium pump of the sarcoplasmic reticulum (SR) (Weber 1968). This view has recently been revised by Endo (1975) who suggests that caffeine potentiates the twitch response, and induces contractures in higher concentrations, by enhancing the calcium-induced calcium release from the SR. Such a mechanism of action does not seem to operate in twitch potentiation by 4-AP as this agent, even in high concentrations (10 mM), fails to induce caffeine-contractures and has no detectable influence on the contractures induced by caffeine (Fig. 6).

From the results presented in Table II it is evident that 4-AP does not cause any detectable change of the resting membrane potential. The maximum rate of rise and the overshoot of the action potential were not significantly different from the control. The rate of decay of the action potential on the other hand was markedly reduced. These changes of the action potential are consistent with previous observations which suggest that 4-AP acts by predominantly decreasing the potassium conductance in frog skeletal muscle fibres (Gillespie and Hutter 1975) and in cockroach (Pelhate and Pichon 1974) and squid (*et al.* 1976) axon membranes and in sympathetic nerves (Kirpekar *et al.* 1977).

In contrast to its effects on the nerve terminal (Lundh *et al.* 1977) 4-AP had a slow onset of action on the muscle fibres, 15–20 min being required for the full effect to appear. Furthermore the effects produced by 4-AP on the action potential and on the twitch were only partially reversible. This is consistent with the idea that there is a very firm bond of 4-AP both to the surface membrane and, in frog muscle, to the sarcoplasmic reticulum inside the fibre (*cf.* above).

It has been suggested that 4-AP increases transmitter release from nerve endings by facilitating calcium influx from the extracellular medium (Molgo *et al.* 1977, Lundh & Theisfeldt 1977). The present results suggest, however, that extracellular calcium is immaterial for the twitch potentiation produced by 4-AP. The enhancement of the isometric twitch

4AP could then be produced when there was no calcium in the extracellular medium, after replacement of calcium in the Ringer solution by lanthanum. It has previously been shown that the presence of lanthanum (Anderson and Edman 1974 a) prevents the membrane depolarization that otherwise occurs as calcium is removed from the bathing fluid (Edman and Grove 1961-1964, Jenden and Reger 1963, Curtis 1963). Lanthanum is able to penetrate the fibre membrane (Lazlo *et al* 1952, Lesseps 1967, Langer and Oak 1972) and causes by itself only a slight twitch potentiation (Anderson and Edman 1974) in the low concentrations (0.05 mM) used here.

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References

- Anderson, K. E. and K. A. P. Edman, Effect of lanthanum on the coupling between membrane excitation and contraction of isolated frog muscle fibres. *Acta physiol. scand.* 1974 90, 113-123.
- Anderson, K. E. and K. A. P. Edman, Effects of lanthanum on potassium conductors of isolated frog muscle fibres of the frog. *Acta physiol. scand.* 1974 b, 90, 124-131.
- Brody, S. A. Some effects of Ca-free choline Ringer solutions on frog skeletal muscle. *J. Physiol. (Lond.)* 1971 261, 73-86.
- Edman, K. A. P. and D. W. Garry, The role of calcium and zinc in the electrical and mechanical responses of frog sartorius muscle. *Experiments (Lond.)* 1961 17, 357.
- Edman, K. A. P. and D. W. Garry, On the role of calcium in the excitation-contraction process of frog sartorius muscle. *J. Physiol. (Lond.)* 1964 170, 139-152.
- Edman, K. A. P. and A. Kessling, The time course of the active state in relation to sarcomere length and movement velocity in single skeletal muscle fibres of the frog. *Acta physiol. scand.* 1971 81, 182-196.
- Edman, K. A. P., D. W. Garry and E. Nilsson, Studies of the excitation-contraction mechanisms in the skeletal muscle and the myocardium. *Physiologists Arch. ges. Physiol.* 1966, 290, 320-324.
- Edman, K. A. P. and S. H. Barry, Excitation-contraction uncoupling in skeletal muscle by dantrolene sodium. *Neuropharmacology Arch. Pharmacol.* 1972, 274, 107-109.
- Edman, K. Mechanism of action of cations on the sarcoplasmic reticulum of skeletal muscle. *Proc. Japan Acad.* 1975 51, 479-484.
- Edman, K. A. P. and O. F. Hotter, The actions of 4-aminopyridine on the delayed potassium current in skeletal muscle fibres. *J. Physiol. (Lond.)* 1973, 252, 70-71P.
- Edman, K. and J. E. Desautels, Effect of dantrolene sodium on calcium movements in single muscle fibres. *Neurophysiol. (Lond.)* 1974, 252, 728-729.
- Edman, K. A. P. and J. F. Rieger, The role of resting potential changes in the contractile failure of frog sartorius muscle during calcium deprivation. *J. Physiol. (Lond.)* 1963, 169, 899-907.
- Edman, K. A. P., M. S. M. Kierulff and J. C. Pra, Effect of 4-aminopyridine on release of noradrenaline from the perfused cat spleen by nerve stimulation. *J. Physiol. (Lond.)* 1977, 272, 517-528.
- Edman, K. A. P. and I. S. Frank, Lanthanum in heart cell culture. Effect on calcium exchange correlated with its localization. *J. Cell Biol.* 1972, 54, 441-455.
- Edman, K. A. P., D. W. Garry, R. Lewin and E. O. Stern, Biological studies on stable and radio active lanthanum compounds. I. On the distribution of lanthanum in the mammalian organism. *J. Nat. Cancer Inst.* 1972, 43, 559-571.
- Edman, K. A. P., Localization by phosphotungstic acid of layer of La staining material external to the cell membrane of myotube cells. *J. Cell Biol.* 1967, 34, 173-183.
- Edman, K. *Paralysis in locomotion after poisoning*. Thesis, University of Lund, 1978.
- Edman, K. and S. Turley, The mode of action of 4-aminopyridine and guanidine on transmitter release from motor nerve terminals. *Europ. J. Pharmacol.* 1977, 42, 411-412.
- Edman, K. A. P. and S. Turley, Antagonism of the paralysis produced by botulinum toxin in the rat. *J. Neurol. Sci.* 1977, 32, 29-43.

- MOLOO, M. J. M. LEMMONAN and P. LECHAT. Modification de la libération de transmetteur à la jonction neuromusculaire de Grenouille sous l'action de l'aminopyridine. *C. R. Acad. Sci. (Paris)* 1973. **276**, D 281-1637-1639.
- MOLOO, J. M. LEMMONAN and P. LECHAT. Effects of 4-aminopyridine at the frog neuromuscular junction. *J. Pharmacol. exp. Ther.* 1977. **203**, 653-663.
- PAIKOV, P. S., E. A. STANOV and V. V. MIROV. New anticholinergic and analeptic drug Pilocarpine and its use in anaesthesia. *Eksp. Khir. Anestezii* 1973. **18**, 48. (In Russian.)
- PELLIAT, M. and Y. PICHON. Selective inhibition of potassium current in the giant axon of the cockroach. *J. Physiol. (Lond.)* 1974. **242**, 90-91P.
- SANDOW, A. and H. PREMER. Muscular contraction as regulated by action potential. *Science* 1964. **144**, 1470-1472.
- SANDOW, A., S. R. TAYLOR and H. PREMER. Role of the action potential in excitation-contraction coupling. *Fed. Proc.* 1965. **24**, 1116-1123.
- SANDOW, A., S. R. TAYLOR, A. ISAACSON and J. J. STROMM. Electrochemical coupling in potentiated muscular contraction. *Science* 1964. **143**, 577-579.
- TAYLOR, S. R., H. PREMER and A. SANDOW. Action potential parameters affecting excitation-contraction coupling. *J. gen. Physiol.* 1972. **59**, 41-436.
- WEBER, A. The mechanism of the action of caffeine on sarcoplasmic reticulum. *J. gen. Physiol.* 1969. **53**, 760-772.
- YEIL, J. Z., G. S. OXFORD, C. H. WU and T. NARAHASHI. Interaction of aminopyridines with potassium channels of squid axon membrane. *Biophys. J.* 1976. **16**, 77-81.

Studies on the enzymatic blood-brain barrier: Quantitative measurements of DOPA decarboxylase in the wall of microvessels as related to the parenchyma in various CNS regions

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Abstract

HÄRBERG, J. E., B. FALCK, CH. ÖWMAN and E. ROSENGRÉN. *Studies on the enzymatic blood-brain barrier: Quantitative measurements of DOPA decarboxylase in the wall of microvessels as related to the parenchyma in various CNS regions*. Acta physiol. scand. 1979; 105: 453-460.

Presence of DOPA decarboxylase in cerebral microvessels (capillaries and venules) impedes the passage of some precursors into the brain. The relative amount of DOPA decarboxylase in this trapping element is compared to the parenchyma *per se* as was estimated in various CNS regions, measuring the release of dopamine from L-DOPA *in vivo* by *in vivo* in two experimental models on rats and rabbits. (1) In the case of the treatment with peripheral decarboxylase inhibitor (carbidopa, which inhibits also intracellular DOPA decarboxylase in the CNS) it could be calculated that the enzyme in the microvessels of the nucleus (rich in catecholamine nerve terminals), cerebellum (poor in catecholamine nerves), spinal cord comprised 25, 91 and 79 per cent, respectively, of the total enzyme activity. (2) Measurement of dopamine turnover in the spinal cord following transection at the midthoracic level (which causes destruction of the catecholamine neurons caudal to the lesion since they are all descending) indicated that the fraction of enzyme as found in the carbidopa model, 71 per cent, of the total tissue decarboxylase activity of the microvessel *in vivo*. The results show that considerable portion of tissue decarboxylase in the *in vivo* point in the nerve-cord *in vivo*, where it represents part of an enzymatic blood-brain barrier element.

The concept of a barrier between the blood and the brain parenchyma originates from the hypothesis by Ehrlich (1885) that vital dyes—when injected systemically—pass freely from the circulation into peripheral tissues, but are excluded from the central nervous system. With the advent of electron microscopy an ultrastructural basis for the blood-brain barrier was suggested in that cerebral vessels are distinguished by the presence of closed tight junctions between adjacent endothelial cells (Reese and Karnovsky 1967). This feature—unique for the central vasculature—along with absence of endothelial fenestrations and the paucity of pinocytotic vesicular transport *across* endothelial cells, constitutes the morphological basis

for the blood-brain barrier. In this way the diffusion of substances from the systemic circulation into the brain is severely limited (for review see Rapoport 1976).

Using fluorometric assay and fluorescence microscopic techniques, a number of biochemical investigations have revealed that there is a specific trapping mechanism for within the cerebral capillary endothelium for amine precursors, such as L-DOPA (L-dihydroxyphenylalanine) and L-5-hydroxytryptophan (Bertler *et al* 1966, Owman 1967, Rosengren 1967, Bartholini *et al* 1971, Hardebo *et al* 1976). This trapping mechanism, which constitutes part of an enzymatic barrier, has essentially three phases: the active transport (Wade and Katzman 1975) of the precursor into the endothelial cell, the decarboxylation of the precursor to the corresponding amine, and finally the amines are metabolized by the enzyme monoamine oxidase.

The degree of decarboxylation capacity can be measured directly in isolated fractionated brain microvessels which are essentially free of neuronal contamination (Hardebo 1977a). The capacity of the enzymatic blood-brain barrier under *in vivo* conditions has been estimated in terms of the break-through of L-DOPA into the brain after systemic administration of increasing doses of the amino acid (Hardebo *et al* 1977b). The present paper reports further quantitative aspects of the microvascular trapping mechanism for L-DOPA in the brain by chemical determinations of the DOPA decarboxylase activity in various CNS regions using two models: either after elimination of the neurons (caudal to a spinal cord transection) or following selective inhibition of the enzyme in the vessels (by carbidopa). At certain dose levels, efficiently blocks DOPA decarboxylase in peripheral tissue and brain microvessels, leaving the enzyme within the brain parenchyma essentially unaffected (Bartholini and Pietscher 1969).

Material and Methods

Animals. The study was performed on 16 female Sprague-Dawley rats, weighing 190–200 g, and 18 albino rabbits weighing 1.5–2.0 kg. The animals had free access to standard pellet food and tap water. The spinal cord in 11 of the rats and in all rabbits was transected in the midthoracic region under brief general anesthesia. The urinary bladder was emptied daily by gentle pressure on the abdomen, and care was taken to prevent deformation of the extremities. The animals were killed under light ether anesthesia 10 days after transection by perfusion of the vascular system with 0.9% saline.

Fluorescence microscopy. Immediately after decapitation, small tissue pieces were dissected out from cerebellar hemispheres, the caudate nucleus, and the spinal cord. They were frozen to the temperature of liquid nitrogen and further processed for monoamine fluorescence histochemistry according to the F. Hillarp method (Björklund, Falck and Owman 1977). The paraformaldehyde used had previously been equilibrated with a 70% humidity. The formaldehyde-induced, histochemically visible fluorophore of noradrenaline (NA), dopamine (DA), and DOPA are indistinguishable under standard conditions: they have the same spectral characteristics and all exhibit a green light under the optical conditions (Björklund *et al* 1972).

Chemical determination of catecholamines. DA was determined fluorometrically either according to Hägggödal (1963) or Anton and Sayre (1964) following homogenization of the tissue in 0.4 N perchloric acid. NA was estimated by the radioenzymatic method described by Cuervo, Haley and Iversen (1977) modified by Coyle and Henry (1973); the tissue was homogenized in 0.1 N perchloric acid containing 0.1 mM EDTA.

Determination of DOPA decarboxylase activity. (1) One mm thick slices of the cerebellum, caudate nucleus and cervical spinal cord were cut with a razor blade and transferred to incubation vials containing Krebs-Ringer buffer solution. The buffer had the following composition (mM): NaCl 118, KCl 4.5, CaCl₂ 2.5, MgSO₄ 7H₂O 1.0, NaHCO₃ 2.5, KH₂PO₄ 1.0, which was added 1 mg/ml glucose, 0.2 mg/ml ascorbic acid and 0.1 mg/ml EDTA. The vials were placed in an incubation bath at 37°C for preincubation

10 min followed by incubation during 20 min in the presence of 0.1 mg/ml L-DOPA. Blanks were run with -DOPA. The buffer solution was continuously aerated with a mixture of 95 O₂ and 5 per cent CO₂ of 2.4. One slice from each incubation was taken for fluorescence microscopy and the rest for chemical determination of DA according to Håggendal (1963).

Tissue pieces from the spinal cord were homogenized on ice and preincubated for 10 min in 0.1 M Tris buffer (pH 7.5) in which pyridoxal-5-phosphate had been added, followed by incubation for 20 min in the presence of 0.1 mg/ml L-DOPA. The incubation was performed at 37°C under aerobic conditions with shaking with nitrogen. Beniker and Rosenkrantz (1959). The incubation was stopped with perchloric acid. Boiled homogenates incubated in -DOPA served as blank. The amount of DA formed was determined according to Håggendal (1963).

There was no statistically significant difference (Student's *t*-test) whether the dopamine formation was measured under aerobic conditions or not, even with extended incubation times from 10 to 45 min. In the following the results are presented both of the two techniques that were used in the DOPA decarboxylase assays.

Experiment 1 Three types of experiments were performed with animals in which the spinal cord had been removed.

1.1 Five rats and 8 rabbits were pretreated with the monoamine oxidase inhibitor nialamide (100 mg/kg i.p.) 30 min before the injection of L-DOPA (30 mg/kg i.p.). All rats and 6 rabbits were killed 90 min later and the others were killed after 60 min. The spinal cord was dissected out and pieces were taken cranial to the lesion for chemical determination of DA.

1.2 Three rats and 4 rabbits were pretreated with nialamide (100 mg/kg i.p.) 90 min before sacrifice. One slice from the spinal cord was taken above and below the level of transection for determination of DA activity under aerobic and anaerobic conditions.

1.3 Spinal cord tissue above and below the lesion was homogenized on ice for determination of DOPA decarboxylase activity under aerobic conditions (3 rats and 4 rabbits) or homogenized in perchloric acid for estimation of MA (2 rats and 2 rabbits).

Experiment 2 DOPA decarboxylase inhibition was carried out. In intact rats 5 rats were pretreated with reserpine (10 mg/kg i.p.) and 4 h later with nialamide (100 mg/kg i.p.), followed after 30 min by carbidozine (100 mg/kg i.p.) which effectively inhibits DOPA decarboxylase in peripheral tissue and brain (Gonon and Pissier 1969). The animals were killed 1 h after the last injection. 5 animals received reserpine and nialamide, but no carbidozine. Slices from the cerebellum, caudate nucleus, and spinal cord were incubated under aerobic conditions for determination of DOPA decarboxylase activity.

Experiment 3 The following compounds were used: 3,4-dihydroxyphenylalanine (Dopa), reserpine (Serpasil, Alkermes), nialamide (Nialmid, Pfizer), *o*-methyltyrosine hydrazine (Carbidopa, MSD), pyridoxal-5-phosphate (PLP, Drug House), bromine sodium (Lilly).

Results and Comments

Fluorescence microscopy of the caudate nucleus from animals treated with reserpine and carbidozine with or without the peripheral decarboxylase inhibitor carbidozine, followed by injection with L-DOPA showed an intense green fluorescence in the parenchyma deriving from the very dense network of dopamine-containing axon terminals (cf. Lindvall and Björk 1974). The general background fluorescence of the parenchyma in the cerebellum and spinal cord was very low and only a small number of isolated green-fluorescent axons known as MA (Olsson and Fuxe 1971; Pickel, Segal and Bloom 1974; Nygren and Olsson 1974) was present in the preparations.

In animals not given carbidozine, a green fluorescence of equally high intensity was seen in the walls of capillaries as well as of small veins in all regions studied (Fig. 1), whereas the walls of parenchymal and pial arteries and arterioles were non-fluorescent. The microvessel fluorescence was weaker in the rabbits (killed 20 or 60 min after administration of L-DOPA) than in the rats (killed 20 min after L-DOPA). Tissues from animals pretreated with carbidozine

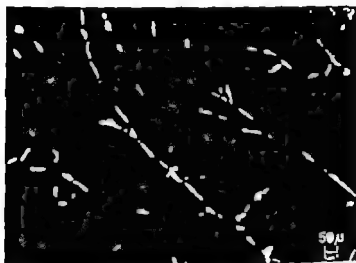


Fig. 1 Spinal cord from a rat pretreated with 10 mg/kg reserpine followed by 100 mg/kg pialamide. 24 h after the injection of 50 mg/kg L-DOPA an intense green fluorescence is seen in the microvessel walls. There is essentially no background fluorescence in the parenchyma. Those of the nuclear regions that are seen in the tissue section represent the largest volume of the pericytes and endothelial cells and are therefore particularly well visible.

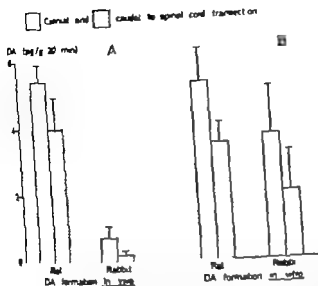
(following reserpine and pialamide as above) and incubated with L-DOPA showed on weak fluorescence in the microvessel wall but, on the other hand, an increased diffuse background fluorescence of the parenchyma.

The density of the network of microvessels was compared in the 3 regions on the basis of the arrangement of intensely fluorescent vessel walls. The density was lower in white than in grey matter but no difference in density was found between the various regions.

Microvessel DOPA decarboxylase activity (carbidopa model) An estimate of the DOPA decarboxylase activity in caudate, cerebellar and spinal cord microvessels was obtained by subtracting the tissue decarboxylase value in animals treated with carbidopa (and thus leaving only central neuronal decarboxylase intact) from the value of animals not given carbidopa (having both neuronal and vascular decarboxylase). Values obtained during incubation without L-DOPA (representing endogenous DA) were subtracted beforehand. The vascular DOPA decarboxylase activity thus calculated was in the cerebellum and spinal cord 2.82 ± 0.50 and $3.12 \pm 0.83 \mu\text{g DA/g tissue} \cdot 20 \text{ min}$, respectively (mean values \pm S.E., Table 1). In the caudate nucleus the mean activity was slightly higher $4.72 \pm 0.78 \text{ ng DA/g} \cdot 20 \text{ min}$. The relative vascular activity comprised 25, 91 and 79 per cent of the total (neuronal + vascular) DOPA decarboxylase activity in the caudate nucleus, cerebellum and spinal cord, respectively. Since the cerebellum and spinal cord contain only few catecholamine-forming neurons it is to be anticipated that a major fraction of the total decarboxylase activity in these regions is represented by the microvessel walls. The situation is reverse in the caudate nucleus which is rich in dopaminergic nerve terminals.

In untreated animals the NA concentration in the spinal cord is about the same in segments cranial and caudal to the midthoracic level (Andén, Magnusson and Rosengren 1965). 7 days after thoracic transection the concentration caudal to the lesion dropped to 13 ± 2 per cent of the concentration cranial to the section in the rat and rabbit, respectively.

DOPA DECARBOXYLASE IN BRAIN MICROVESSELS



DOPA decarboxylase activity (mean values \pm S.E.) expressed as μg dopamine (DA) formed per g wet weight tissue (wet weight) cranial and caudal to cord transection in the rat and rabbit. A. Formalin-treated rats (100 mg/kg i.p.) followed by L-DOPA (50 mg/kg i.p.) 20 min before sacrifice. B. Rats pretreated with reserpine (10 $^{-6}$ M) 20 min, followed by incubation in L-DOPA (50 mg/kg i.p.) 20 min.

cranial DOPA decarboxylase activity (spinal cord transection model). The degree of lesion obtained *in vivo* and *in vitro* cranial and caudal to the midthoracic transection in rat and rabbit are shown in Fig. 1. In non-operated animals, the DA formation is about the same cranial and caudal to the transection level (Andén *et al* 1965). However after transection the decarboxylase activity in the cranial portion does not differ significantly from activity in the corresponding region of non-operated animals (Andén *et al* 1965). In the present study a mean fall in activity of 29 per cent was seen in the rat after transection. Le-DOPA decarboxylase activity present in microvessel walls (and in perivascular sympathetic nerve) could constitute about 70 per cent of the total activity in the spinal cord (cf. the value

Table 1. DOPA decarboxylase activity in various CNS regions of the rat separately expressed in terms of enzyme activity in the parenchyma (neurons) and in the microvessel walls

Brain activity	A: total activity ("neuron + microvessel")	B: activity after reserpine ("neuron")	A minus B ("microvessel activity")
Caudate nucleus	18.91 \pm 1.78	14.19 \pm 1.55	4.72 \pm 0.78
Corpus striatum	3.19 \pm 0.46	0.28 \pm 0.04	2.82 \pm 0.50
Spinal cord	3.95 \pm 0.65	0.83 \pm 0.23	3.12 \pm 0.83

The decarboxylase activity is expressed as the amount of dopamine synthesized in tissue slice (μg formed per g tissue during 20 min) from animals pretreated with reserpine (10 mg/kg i.p.) and methyldopa (100 mg/kg i.p.). The values reflect the level of endogenous dopamine; these values have been subtracted in the calculation of neuronal and microvessel decarboxylase activity. Figures show mean values \pm S.E.M., number of animals 3.

calculated after vascular decarboxylase inhibition) The corresponding value in the rat was considerably lower about 45 per cent.

Discussion

The mechanism that involves decarboxylation and subsequent degradation of cerebral monoamine precursors at the level of the blood brain barrier (Bertler *et al* 1966, Hardebo *et al* 1976) is of interest both from a general biological point of view (see Rapoport 1976) and clinically in the medical treatment of Parkinson's disease (Cotzias, Papavasiliou and Gell 1969). It has previously been shown that the decarboxylation capacity in the wall of brain microvessels has a limit (Hardebo *et al* 1977b) which means that the amine precursor (L DOPA) is able to penetrate into the brain parenchyma at particularly high circulating concentrations. This limit was estimated in a model where increasing doses of L DOPA were given systemically to rats, and the break through of L DOPA into the brain parenchyma at the enzymatic blood brain barrier was estimated by comparing the amount of newly formed DA in brain structures with and without decarboxylase-containing neurons (the cerebellum and cerebellum, respectively). The microvessels were found to efficiently trap L DOPA in their walls up to a circulating level corresponding to an i.p. dose of 100 mg/kg. It could be estimated that approximately 3 per cent of the exogenous L DOPA is decarboxylated at the level of the blood brain barrier whereas the remainder of the conversion takes place in peripheral tissues (Hardebo *et al* 1977b).

In the present study the efficiency of the trapping mechanism was investigated more directly by measuring the decarboxylating capacity in the microvessel wall of the cerebral parenchyma. The major problem encountered was to separate that portion of the decarboxylation taking place in the microvessels from the amine formation occurring in the monoaminergic neuron systems. Two approaches were used: one in which the microvascular decarboxylase was inactivated by carbidopa and another in which the neurons were selectively eliminated by transection of the spinal cord leaving the microvascular decarboxylating capacity intact.

The decarboxylase inhibitor carbidopa (α -methyldopa hydrazine), is selective in the sense that it, probably due to a poor penetration through the blood brain barrier, leaves neuronal decarboxylase in the central nervous system unaffected at certain dose levels which only inhibits peripheral DOPA decarboxylase including that in the brain microvessels (Bartholini and Pietscher 1969). The effect of carbidopa was checked by fluorescence microscopy. Thus, incubation of brain tissue in the presence of L DOPA produced an intense fluorescence (which has been shown to be represented mainly by dopamine; Bertler *et al* 1966) in the microvessel walls that, on the other hand, was almost absent in tissues from carbidopa-treated animals. Persisting decarboxylase activity in the neuronal system (in reserpine-pretreated animals) was indicated by an equally high neuronal fluorescence after L DOPA incubation, whether the animals had received carbidopa or not. It was therefore assumed that most—if not all—of the decarboxylase activity measured in preparations from carbidopa-treated animals reflected neuronal enzyme activity. This was corroborated by the finding that the neuronal decarboxylase thus calculated comprised a much higher fraction

of decarboxylase in the caudate nucleus than in the cerebellum and spinal cord, which have a much lower number of catecholaminergic neurons. In fact, the relationship between decarboxylase activity measured in the brain parenchyma of carbidopa-treated animals corresponds very well to the relative concentrations of catecholamines in the spinal cord, caudate and caudate nucleus, respectively (see catecholamine figures compiled by Holtz and Stjärnman 1972).

The monoamine neuron system in the spinal cord is entirely descending, which means that transection produces an almost complete disappearance of NA caudal to the lesion (Andén *et al.* 1965). It can thus be predicted that remaining catecholamine formation occurs in the descending vessel walls (and probably to a minor extent, in perivascular adrenergic nerves arising in the spinal cord segmentally). After transection, the DOPA decarboxylase activity in the caudal portion of the cord dropped by 79% in the rat, which would indicate that approximately 70% of spinal cord DOPA decarboxylase activity resides in the vascular space. This figure agrees well with the value (79%) calculated from the carbidopa model. The corresponding figure for the intraspinal vascular DOPA decarboxylase activity in the rabbit was lower, only 45%, compared with the rat values. This is in good agreement with the lower enzyme activity that is reflected by a lower wall fluorescence in the microvessels of rabbits under conditions equal to those for rats. The corresponding figure in rabbit experiments presented by Andén *et al.* (1965) are somewhat lower.

In the previously mentioned *in vitro* model, in which the decarboxylating capacity in the vessel walls was estimated after i.p. administration of increasing doses of L-DOPA (to animals pretreated with reserpine and the monoamine oxidase inhibitor nialamide), it was found that the maximum amount of DA formed in the cerebellar vessels corresponded to a concentration of 3.12 µg/g during 20 min. The correlating value for the *in vivo* formation calculated from the carbidopa model (see Table I) was very close, 2.82 µg/g formed during 20 min, showing a good agreement between the two different model approaches. A slight difference was found with the carbidopa model, in terms of microvascular DA formation, being the order caudate nucleus > spinal cord > cerebellum. The same order of effect has been found when comparing the decarboxylase activity in isolated fractions of homogenate from the caudate nucleus and cerebellum in various animals (Hardebo *et al.*, 1977), and it was also indicated by the cytofluorometric measurements of the formaldehyde released capillary wall fluorescence in rats given L-DOPA (Hardebo *et al.*, 1977b).

Previously high doses of L-DOPA have been given in the medical treatment of Parkinson's disease due to the decarboxylation activity in the peripheral tissues and the efficiency of the blood-brain barrier to impede the passage of L-DOPA into the brain, with frequent side-effects as result. Based on this knowledge, including the decarboxylating capacity of the blood-brain barrier (Bertler *et al.*, 1966, Bartholini *et al.* 1971), it has now been possible to overcome this problem: a decarboxylase inhibitor effective in peripheral tissues as well as in the capillary walls but not in the brain parenchyma, has been added to L-DOPA in the drug treatment of Parkinson's disease. This means that the amine precursor can now be administered in lower doses, reducing the incidence of side-effects.

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Effects of vasoactive intestinal polypeptide on blood flow motility and fluid transport in the gastrointestinal tract of the cat

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Abstract

IRVING S. M. JODAL, O. LUNDQVIST and A. SÖQVIST *Effects of vasoactive intestinal polypeptide on blood flow motility and fluid transport in the gastrointestinal tract of the cat.* Acta physiol. scand. 1979 105 461-468.

Effects of dose dependent infusions of vasoactive intestinal polypeptide (VIP) on gastric motility, small intestine blood flow and colonic motility were studied in the cat. Regional blood flow was also followed. All experiments in the stomach VIP produced a gastric relaxation and a blood flow increase. The gastric response was smaller to that observed when eliciting the vago-vagal reflex relaxation by distending the oesophagus. In the small intestine hyperaemia and decrease of net water uptake was observed. The slowing small intestine of VIP decrease of net water uptake was seen without any change of local blood flow. Large amounts of VIP produced transient secretory state in the small intestine. Intestinal hyperaemia was seen immediately upon starting the infusion of the drug. After 2-3 days of continuous treatment of the cat was apparent. The administration of atropine to the animal did not clearly affect any of the responses produced by VIP. The results are discussed in relation to VIP as a neurotransmitter in the gastrointestinal tract.

Vasoactive intestinal polypeptide (VIP), a compound with a large spectrum of biological actions, was originally isolated from the gastrointestinal tract (Said and Mutt 1970). The molecular mass of VIP exceeds 3 000 d and it is composed of 28 amino acids. The sequence of the amino acids is fully established and it resembles glucagon and secretin. These well established hormones produce biological effects similar to VIP. It has therefore been labelled 'intestinal hormone' together with several other peptides recently isolated from the gastrointestinal tract (Grossman *et al.* 1974).

Immunohistochemical methods for studying the tissue localization of VIP have, however, demonstrated that most, if not all, VIP is localized to nervous tissue not only in the stomach but also in peripheral organs (see *cf.* Larsson *et al.* 1976). Furthermore, immunohistochemical and biochemical techniques show that VIP is predominantly localized to neurons (Giachetti *et al.* 1977 Larsson 1977 Emsen *et al.* 1978), suggesting that VIP may be a neurotransmitter. This proposal was further strengthened by the demonstration of VIP release from the alimentary canal upon direct electric stimulation of the vagal and

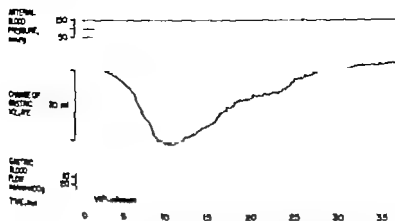


Fig. 1. The effects of close infusion of VIP on arterial blood pressure, gastric blood flow and gastric volume. VIP is infused at rate of 3.4 $\mu\text{mol/min}$ increasing plasma concentration 0.67 $\mu\text{mol/l}$. A relative fall of the stomach reflected as downward deflection of the gastric volume recording.

Fig. 3-10 on long colonic segment for the experiment. The colonic vein was cannulated and blood was recorded continuously by drop recorder unit operating an ordinate writer as described for the rat. Autonomic nervous influences on the colon were eliminated by cutting the pelvic splanchnic nerve and the abdominal aorta.

After surgery is followed by recording intraluminal osmotic changes with osmotic transducer placed in one end of the colonic segment, the other end being closed. Its ligature, intraluminal pressure was at 2-4 cm H_2O .

The intraluminal infusions of VIP were made into side-branch to the colonic artery.

Isotonic and drops

The isotonically infused buffer solution contained 10 mmol NaHCO_3 in 100 ml 10% glucose solution. It was infused at rate of 0.1-0.2 ml/min.

The solution used to perfuse the lumen of the small intestine contained (mmol/l): NaCl 122, KCl 3.3, KH_2PO_4 1.2, MgCl_2 0.6, CaCl_2 2.5, glucose 30. The osmolality of the solution was 300 mOsm/kg H_2O .

VIP was dissolved in physiological saline at various concentrations. The infusion rates used are given in Table 1. VIP was obtained from Dr V. Mutt at the Gastrointestinal Research Unit, Karolinska Institute, Stockholm, Sweden.

Isoproterenol (0.3-1 $\mu\text{g/kg}$ b.w.) was administered during the course of the experiments.

Calculations

The increase of arterial plasma VIP concentration produced by the i.a. infusion of the drug was calculated by using the infusion rate ($\mu\text{g/min}$) by the plasma flow (ml/min) as measured. Since the infusion was into the small intestine, slight overestimation of the increase of VIP concentration since blood flow is increased upon infusion. However, recirculation tends to increase plasma drug concentration during longer infusions.

Experiments on the stomach

1) Extra VIP was infused close i.a. to the stomach, usually during a 3 min period, and at rates between 2 and 12 $\mu\text{mol/min}$ (7-42 $\mu\text{g/min}$) which led to plasma VIP concentrations between 0.2 and 3.0 $\mu\text{mol/l}$. Changes in total gastric blood flow and gastric volume were recorded. Fig. 1 illustrates a typical experiment. Upon infusing VIP a small increase of

Results

pelvic nerves, and also when various types of reflexes are induced via these nerves (Fahkrug *et al* 1978 a, b)

One of the criteria for a neurotransmitter is that it should elicit the same response in an organ when administered intravascularly as when released from nervous endings. However there is a paucity of data concerning the effects *in vivo* of intravascularly administered VIP on gastrointestinal function particularly with respect to motility. Thus, in the present study the effect of intravascularly infused VIP on motility, blood flow and water absorption in the gastrointestinal canal *in situ* was investigated.

Methods

Animals and general operative procedures

The experiments were performed on 14 cats, anaesthetized with chloralose (50 mg/kg b.w.) and ether. The cats had been deprived of food for at least 24 h with free access to water. They exhibited no obvious signs of gastrointestinal disease.

The following procedures were common to all types of experiments performed in this study. A midline incision was made in the abdomen by a midline incision, the great omentum and the spleen were extirpated. The femoral artery was cannulated after heparinization (3–5 mg/kg b.w. i.v.) to record mean arterial blood pressure by means of a Statham pressure transducer (model P23AC). All measured parameters (see below) were recorded on a Grass polygraph. The splanchnic nerves were cut bilaterally.

A slow intravenous infusion of a glucose solution containing bicarbonate (see below) was started at the time of the induction of anaesthesia and then continued throughout the experiment. This infusion has previously been shown to maintain arterial pH at a normal level, despite varying degrees of operative trauma (Haglund and Lundgren 1972).

Experiments on the stomach

A loose ligature was placed around the cardia without damaging the vagal nerve trunks. A 16-gauge plastic catheter was introduced into the stomach at the pylorus through a duodenostomy and connected to a volume transducer for continuous recording of changes in gastric volume. The stomach and the volume recording system was filled with isotonic saline and arranged in such a way that the intragastric pressure could be set and continuously adjusted to the desired level, usually 2 cm above that at the pylorus.

Gastric blood flow was registered via a catheter in the splenic vein connected to a drop recorder operating an ordinate writer. All other veins draining the stomach were ligated. Intra-arterial infusion of VIP to the stomach was made into the hepatic artery in a retrograde direction. A vago-vagal receptor relaxation of the stomach was elicited effectively by distension of the esophagus (Abrahamson and Jost 1969). This was achieved by filling a small balloon, placed in the proximal part of the esophagus, with 20 ml saline.

Experiments on the small intestine

An 8–10 cm long jejunal segment was isolated with its blood supply intact. The colon and remaining part of the small intestine were extirpated. The jejunal segment was so chosen that its arterial supply was derived from one single main branch of the superior mesenteric artery and i.a. infusions of VIP were in most experiments performed in an adjacent branch. Total venous outflow from the jejunal segment and its tributaries was registered via a catheter in the superior mesenteric vein, as described above.

Net intestinal transport of fluid was measured with the method described in detail by Jodal *et al.* (1971) where the intestinal lumen was perfused with a body warm Krebs-glucose solution (see below) at a rate of 1 ml/min in a recirculating system. Changes in motility and/or net fluid transport were registered by a volume transducer connected to the system via a T tube.

Experiment on the colon

In these experiments the small intestine was extirpated and the inferior mesenteric artery was divided. This procedure has been shown not to decrease blood flow to the feline large bowel (Hultén *et al.* 1971) which is simply supplied in the superior mesenteric artery. The distal part of the colon was extirpated.

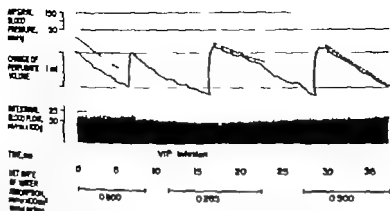


Fig. 3. The effects of three infusions of VIP on arterial blood pressure, intestinal blood flow and jejunal motility. The drug is infused at rate of 0.017 nmol/min increasing plasma concentrations 3 and 1. The upward deflections of the recording of perfusate volume were produced by adding 1 ml of saline to the volume transducer.

Intestinal motility it was calculated that jejunal blood flow increased 40–50% above control when increasing arterial plasma VIP concentrations between 0.15 and 0.45 $\mu\text{mol/L}$. Arterial administration had no appreciable effects on the intestinal responses to VIP.

Effects of VIP on the colon

15 μg VIP was infused i.a. to the colon while colonic blood flow and motility were recorded. The infusions were performed at rates varying between 1.7 and 7.1 nmol/min (0.11–0.44 $\mu\text{g}/\text{min}$), increasing plasma VIP concentrations between 0.35 and 4.6 $\mu\text{mol/L}$. A representative experiment is shown in Fig. 4. Upon starting the VIP infusion a marked blood flow increase was immediately caused, but subsided after about half a minute and levelled off at a moderate flow elevation maintained throughout the infusion. After about 2 min a contraction of the colonic muscularis occurred, reflected as an increased fluid volume in the volume transducer. When the VIP infusion was stopped blood flow returned rapidly to control level while the colonic volume increase returned to control first 5–10 min later.

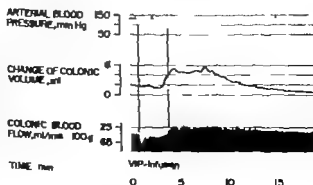


Fig. 4. The effects of three infusions of VIP on arterial blood pressure, colonic blood flow and colonic motility. VIP was infused at rate of 3.4 nmol/min increasing plasma concentrations 1.2 and 1. A contraction of the colonic muscularis occurred, reflected as an upward deflection of the colonic volume transducer.

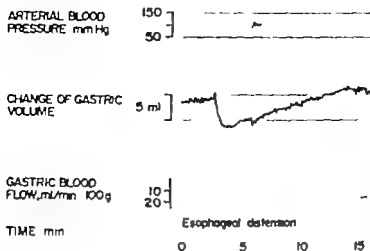


Fig. 1. The effects of distend the esophagus on arterial blood pressure, gastric blood flow and gastric motility. The experiment was performed on the same animal as illustrated in Fig. 1.

gastric blood flow was observed concomitant to a pronounced increase in gastric volume depicted as a downward deflection in the recording. Blood flow increased somewhat during the period of constant VIP infusion and returned to control fairly quickly after the infusion. The changes in gastric volume exhibited a characteristic, fairly fast increase during the period of drug infusion, while the return to control volume was quite slow, lasting 20–30 min. A closely similar change of gastric volume could be elicited reflexly by distending a balloon in the esophagus, as illustrated in Fig. 2 taken from the same experiment. No significant change in gastric blood flow was observed upon esophageal distension.

In 2 experiments, an attempt was made to estimate the increase of arterial plasma VIP concentration necessary to produce a gastric relaxation of the same magnitude as that elicited by distension. It was found to be 0.3 and 0.5 $\mu\text{mol/l}$ plasma.

The motility effects observed upon VIP infusions were similar in magnitude before and after giving atropine to the animal.

Experiments on the small intestine

The effects of intraarterial VIP infusions on blood flow and net water uptake in the small intestine were studied in 7 cats. VIP was infused at a rate of 0.001–7.1 nmol/min (0.001– $\mu\text{g/min}$) increasing the plasma VIP concentration 0.4–1900 nmol/l. The high infusion rates were usually maintained for only 3 min to study the VIP effects on blood flow. At low rates of infusion the experimental runs were prolonged for 10–15 min to allow recordings of VIP effects on net fluid transport rate in the intestine. Plasma concentrations of VIP were calculated without taking into consideration recirculation of the peptide (see Methods). Fig. 3 illustrates a representative experiment, showing that blood flow is slightly increased upon VIP infusion while the rate of intestinal water uptake is reduced as indicated by dotted line.

The lowest increase of arterial plasma VIP concentration able to decrease intestinal net fluid uptake was 2.0 nmol/l, which did not significantly increase total intestinal blood flow. At high rates of infusion, increasing plasma VIP concentrations above 0.5 $\mu\text{mol/l}$, a marked intestinal secretion was observed continuing also after ended infusion with a slow return to control rate of absorption first about 30 min after the infusion period. With regard to

these concentrations is, indeed, to be expected if VIP is a neurotransmitter in the gastrointestinal tract. VIP is a fairly large, water soluble compound suggesting that its capillary permeability is fairly low (Landis and Pappenheimer 1963). Thus VIP when released topically from nerve endings in the tissues, will initially show a much restricted tissue distribution. The compound will then be locally dispersed by diffusion and probably only to a small area reach the blood stream due to the hindrance offered by the capillary walls. Furthermore, potent mechanisms are likely to exist at synaptic clefts to degrade the VIP molecule or to trap it back into the nerve endings. Similarly the low capillary permeability of VIP necessitates a high rate of intramural infusion of the compound to reach the same VIP concentration at the effector cell as that induced by the nervous release. Such principles may, for instance, for the blood concentration of neurogenically released noradrenaline and intramurally infused noradrenaline when given in concentration to match the neurogenic effects (Ljung 1970).

Some of the nervous effects described above were studied with regard to their effects on intestinal fluid transport. In the present study it was observed that one could induce a decrease of net fluid absorption in the small intestine at plasma VIP concentrations well above those inducing a vasodilatation. In fact, the increase of arterial plasma VIP concentration that diminished net fluid uptake was only 5-10 times greater than the increase of venous plasma concentration observed upon mechanical stimulation of the intestinal mucosa (Fährberg *et al.* 1978 b). It seems therefore reasonable to conclude that VIP profoundly affects intestinal net fluid transport rate at very low plasma concentrations, possibly even at such plasma concentrations seen during physiological circumstances. This conclusion is supported by the findings reported from studies on isolated intestinal epithelial cells indicating the presence of membrane receptors with a very high affinity to VIP (Dupont *et al.* 1973, Laborde *et al.* 1978).

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References

- BRUNSTEDER, H. and G. LUNDHOLM, Electrical reflex vagal relaxation of the stomach from pharyngeal myoelectric activity in the cat. *Acta physiol. scand.* 1969 77 172-178.
- BRUNSTEDER, G. O. and M. O. GÖRANSSON, Intestinal secretion stimulation by peptides. *Science* 1971 174 124-125.
- BRUNSTEDER, G. O. and J. SVENSSON, Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut. *Acta physiol. scand.* 1971 82 177-190.
- BRUNSTEDER, G. O., M. LARSSON and G. RÖNNELID, Isolation of epithelial cells of human colon and demonstration of the action of VIP. *Scand. J. Gastroenterol.* 1978 13, Suppl. 49-51.
- BRUNSTEDER, G. O., J. FÄHRBERG, O. B. SCHAFFALITZKY DE MUCKADILL, T. M. JENSELL and L. L. IVERSEN, Porcine intestinal polypeptide (VIP): vascular localization and potassium evoked release from rat jejunal villi. *Brain Res.* 1978 143 174-178.
- BRUNSTEDER, G. O., H. CLARKE, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADILL, Influence of the enteric nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *J. Physiol. (Lond.)* 1978 282 405-422.
- BRUNSTEDER, G. O., U. HAGLUND, M. JODAL, O. LUNDGREN, L. OLSSON and O. B. SCHAFFALITZKY DE MUCKADILL, Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tract of cats: possible physiological implications. *J. Physiol. (Lond.)* in press.

To quantitate the VIP effects on colonic blood flow the increase of arterial plasma VIP concentration needed to produce an initial doubling of blood flow (Fig. 4) was determined. It varied between 1 and 4 $\mu\text{mol/l}$ plasma.

Atropine had no effect on the VIP induced changes in colonic blood flow and motility. In 3 of the 5 colon expts. Isoprenaline and papaverin were infused i.a. in the same way as for VIP. Vasodilatations of the same magnitude as with VIP were then produced but colonic contractions were never observed. If anything, minor relaxations occurred.

Discussion

In the present study the effects of i.a. infused VIP on various gastrointestinal functions have been investigated. In the stomach it was possible to demonstrate that VIP relaxed both the vascular smooth muscle cells and the gastric muscularis propria. Relaxatory effects of VIP on gastric smooth muscle has been reported *in vitro* (Piper, Said and Vane 1970). In contrast to the vascular effects the gastric relaxatory response to VIP under *in vitro* conditions was very longlasting returning to control first 20–40 min after ended infusion. This response closely mimics the receptive relaxation elicited by direct stimulation of the efferent vagal relaxatory fibres (Martinson 1965) or by their reflex activation by way of distending the esophagus (Abrahamsson and Jansson 1969, Fig. 2). In the small intestine VIP induced vasodilatation and a decrease of net water transport. At high VIP concentration intestinal secretion was recorded. These observations are in agreement with earlier reports (Blood flow: Said and Mutt 1970, Thulin and Olsson 1973, Kachelhoffer *et al.* 1974, Net water transport: Berbezat and Grossman 1971, Krejs *et al.* 1978). Finally in the colon a hyperemia and a delayed contraction was recorded upon VIP infusion. These two colonic responses are similar to those induced by pelvic nerve stimulation in animals given atropine (Hobbs and Jodal 1969).

In an earlier report from this laboratory (Fahrenkrug *et al.* 1978 b) it was shown that VIP was released from the gastrointestinal tract when eliciting three different non-adrenergic, non-cholinergic neurogenic responses, *i.e.* the vagally mediated gastric receptive relaxation (Martinson 1965), the intestinal vasodilatation produced by mechanical stimulation of the intestinal mucosa (Biber *et al.* 1971) and the colonic hyperemia evoked by the pelvic nerves (Hultén *et al.* 1969). From these and other observations it was proposed that VIP is a neurotransmitter in the gastrointestinal tract. The present results strengthen this conclusion by showing that the intravascular infusion of the proposed neurotransmitter evokes physiological effects closely similar to those seen when the mentioned non-adrenergic non-cholinergic nervous mechanisms of the alimentary canal are activated.

In the study by Fahrenkrug *et al.* (1978 b) the increase of venous plasma VIP concentrations, accompanying the induction of the different nervous effects, were determined. In the present study the arterial VIP concentrations necessary for producing the same gastrointestinal effects were estimated. A comparison of the plasma VIP concentrations in the two experimental situations reveals that the nervous release of VIP into the blood stream was only 1/10 000 of the arterial VIP concentrations needed to induce quantitatively similar physiological effects upon intraarterial VIP administration. This large difference between

same concentrations is, indeed, to be expected if VIP is a neurotransmitter in the gastrointestinal tract. VIP is a fairly large, water soluble compound suggesting that its capillary permeability is fairly low (Landis and Pappenheimer 1963). Thus VIP when released topically from nerve endings in the tissues, will initially show a much restricted tissue distribution. The compound will then be locally dispersed by diffusion and probably only to a small extent reach the blood stream due to the hindrance offered by the capillary walls. Furthermore, potent mechanisms are likely to exist at synaptic clefts to degrade the VIP molecule and/or bring it back into the nerve endings. Similarly the low capillary permeability of VIP necessitates a high rate of intraarterial infusion of the compound to reach the same VIP concentration at the effector cell as that induced by the nervous release. Such principles hold, for instance, for the blood concentration of neurogenically released noradrenaline and intraarterially infused noradrenaline when given in concentration to match the neurogenic effects (Ljung 1970).

None of the nervous effects described above were studied with regard to their effects on mucosal fluid transport. In the present study it was observed that one could induce decrease of net fluid absorption in the small intestine at plasma VIP concentrations well below those inducing a vasodilatation. In fact, the increase of arterial plasma VIP concentration that diminished net fluid uptake was only 5–10 times greater than the increase of venous plasma concentration observed upon mechanical stimulation of the intestinal mucosa (Fahrenkrug *et al.* 1978 b). It seems therefore reasonable to conclude that VIP profoundly affects intestinal net fluid transport rate at very low plasma concentrations, possibly even at such plasma concentrations seen during physiological circumstances. This conclusion is corroborated by the findings reported from studies on isolated intestinal epithelial cells indicating the presence of membrane receptors with a very high affinity to VIP (Dupont *et al.* 1978, Lamberth *et al.* 1978).

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References

- BERGLUND, H. and G. LUNDGREN, Elimination of reflex vagal relaxation of the stomach from pharynx and oesophagus in the cat. *Acta physiol. scand.* 1969 77 172–178.
- BRILL, G. O. and M. O. GROSSMAN, Intestinal secretion stimulation by peptides. *Science* 1971 174 12–13.
- DAHL, B. G. LINDGREN and J. S. VYSE, Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut. *Acta physiol. scand.* 1971 82 177–190.
- DAHL, B. G., M. LAMBERTH and G. ROSSIGNOL, Isolation of epithelial cells of human colon and demonstration of the action of VIP. *Scand. J. Gastroenterol.* 1978 13 Suppl. 49–51.
- DAHL, B. G., J. FÄRNEBERG, O. B. SCHAFFALITZKY DE MUCKADELL, T. M. JENSEN and L. L. IVERSEN, Vasoactive intestinal polypeptide (VIP): vascular localization and potently evoked release from rat jejunum. *Bras. Rev.* 1978 143 174–178.
- FÄRNEBERG, J., R. GALLI, J. J. HOLST and O. B. SCHAFFALITZKY DE MUCKADELL, Influence of the autonomic nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *J. Physiol. (Lond.)* 1978 280 405–422.
- FÄRNEBERG, J., U. HAGLUND, M. JENSEN, O. LUNDGREN, L. OLSSON and O. B. SCHAFFALITZKY DE MUCKADELL, Nervous release of vasoactive intestinal polypeptide in the gastrointestinal tract of cats: possible physiological implications. *J. Physiol. (Lond.)* 1978, in press.

- GIACHETTI, A. F. KONIGS and S. I. SAID, VIP localization in synaptosomes of rat brain. *Fed. Proc.* 1971 30, 951.
- HAGLUND, U. and O. LUNDÖREN, Reactions within consecutive vascular sections of the small intestine of the cat during prolonged hypotension. *Acta physiol. scand.* 1972, 84, 151-163.
- HULTÉN, L. and M. JODAL, Extrinsic nervous control of colonic motility. *Acta physiol. scand.* 1969, Suppl. 335, 21-38.
- HULTÉN, L., M. JODAL and O. LUNDÖREN, Extrinsic nervous control of colonic blood flow. *Acta physiol. scand.* 1969, Suppl. 335, 39-50.
- JODAL, M., D. A. HALLBÄCK, J. SVANVIG and O. LUNDÖREN, A method for the continuous study of water transport in the feline small bowel. *Acta physiol. scand.* 1975, 95, 441-447.
- KACHELHOFFER, J., M. R. ELOY, A. POUSSE, M. HOHMATTER and J. F. GRENIER, Mesenteric splanchnic effects of vasoactive intestinal polypeptide study on perfused isolated canine jejunal loops. *Pflügers Arch. Physiol.* 1974, 332, 37-46.
- KREIS, G., J. R. M. BARKLEY, N. W. READ and J. S. FORDTRAN, Intestinal secretion induced by vasoactive intestinal polypeptide. *J. clin. Invest.* 1978, 61, 1337-1345.
- LABURTIN, M., M. TOUCHET, C. BOSSARD, O. CHEVALIER, A. ZWETBAUM and G. ROBELLY, Vasoactive intestinal peptide (VIP) stimulates cyclic AMP accumulation in human malignant intestinal cell lines in cultures. *Scand. J. Gastroenterol.* 1978, 13, Suppl. 49, 107.
- LARSSON, L. I. Ultrastructural localization of a new neuronal peptide (VIP). *Histochem.* 1977, 34, 173-176.
- LARSSON, L. I., J. FAHRENKRUG, O. SCHIAFFALITZKY DE MUCKADILL, F. SUNDLER, R. HÅKANSSON and J. F. REHFELD, Localization of vasoactive intestinal polypeptide (VIP) in central and peripheral nerves. *Proc. nat. Acad. Sci.* 1976, 73, 3197-3200.
- LIUNG, B., Nerves and myogenic mechanisms in the control of a vascular neuroeffector system. An integrated survey of a series of studies on the portal vein. *Acta physiol. scand.* 1970, Suppl. 349, 33-61.
- MARTINSON, J. Studies on the efferent vagal control of the stomach. *Acta physiol. scand.* 1963, 61, Suppl. 255.
- PIPER, P. J., S. I. SAID and J. R. VANE, Effects on smooth muscle preparations of unidentified splanchnic peptides from intestine and lung. *Nature (Lond.)* 1970, 225, 1144-1146.
- SAID, S. I. and V. MUTT, Polypeptide with broad biological activity: Isolation from small intestine. *Science* 1970, 169, 1217-1218.
- SAID, S. I. and V. MUTT, Potent peripheral and splanchnic vasodilator peptide from normal gut. *Nature (Lond.)* 1970, 225, 863-864.
- THULIN, L. and P. OLSSON, Effects of intestinal peptide mixture C12 and vasoactive intestinal peptide V1B on splanchnic circulation in the dog. *Acta chir. scand.* 1973, 139, 691-697.

Vascular anatomy and tissue osmolality in the filiform and fungiform papillae of the cat's tongue

By

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Abstract

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The vascular anatomy of the filiform and fungiform papillae of the feline tongue was studied by i.a. injection of latex ink. Vascular loops of various appearances were found in the types of papillae studied, *i.e.* large and the small filiform papillae and the fungiform ones. Such hairpin loops may function as countercurrent exchangers and to test this hypothesis tissue osmolality was determined in the papillae, alone exposing them to various isotonic electrolyte solutions. The large filiform papillae with vascular arrangement similar to that of intestinal villi exhibited marked osmolar gradient from tip to base when exposed to solutions containing both glucose and sodium. If sodium and/or glucose was excluded from the solution, tissue osmolality was significantly decreased. This was also the case when the chloride ions of the solution was substituted with sulphate. The small filiform papillae are only provided with one or few capillary loops. They exhibited less marked osmolar gradient than the large ones and some of the different electrolyte solutions decreased the gradient. In the fungiform papillae a tissue hyperosmolality at the tip was also demonstrated. It is proposed that the papillary epithelium is provided with active transport mechanisms and that the papillary vessels function as countercurrent exchangers. The functional importance of these mechanisms are tentatively discussed.

The presence of a countercurrent exchanger in intestinal villi has been demonstrated (e.g. Bond *et al.* 1977, Hallbäck *et al.* 1978 a, Lundgren 1967, 1974). The anatomical basis for this mechanism are the hairpin vascular loops formed by a central artery and the surrounding dense network of subepithelial capillaries. The main direction of blood flow in these two limbs is opposite, and since the intervacular distance between the limbs of the loop is small (about 20 μ m) and the blood transit time is relatively great (4-6 s), it is possible for easily diffusible substances to diffuse from the artery to the capillaries or vice versa. If an easily diffusible compound approaches the exchanger from the luminal side, it is thus possible for this solute to diffuse from the capillaries to the artery delaying its net blood absorption (Lundgren 1967, Svanvik 1973). Furthermore, by acting as a countercurrent multiplier, a hyperosmolar tissue region is established at the villous tip (Hallbäck *et al.* 1971, Jodal *et al.* 1978) in a way similar to that described in the renal medulla.

The papillae on the mammalian tongue seem to have a vascular arrangement resembling that of the intestinal villi at least to judge from studies performed on the dog (for review see Hellkant 1972). Thus, a countercurrent exchanger may exist also in these papillae as the present study was initiated to test this hypothesis. Its purpose was twofold. First, it was deemed necessary to investigate the cat with regards to its vascular anatomy in the papilla of the tongue since such studies are largely lacking. Second experiments were performed using a cryoscopic technique, to establish the possible presence of a hyperosmolar region in the papillae, which would indicate the existence of a countercurrent exchanger.

A preliminary abstract of this report has been published (Hallbjäck *et al.* 1978 b).

Materials and methods

A. Experimental procedures

The studies were performed on cats anesthetized with chloralose (50 mg/kg b.w.) after induction. The cats were heparinized and the right femoral artery was cannulated to record blood pressure by a pressure transducer (Statham P23AC) connected to a Grass polygraph. Cats having a mean arterial blood pressure below 90 mmHg were not included in the study.

The tongue placed in a little plastic cup *in vivo* was exposed to an electrolyte solution of known composition by dripping the solution on the dorsum of the tongue for about 30 min at a rate of 1 ml/min. In most of the experiments the solution was heated to a temperature of 36–38°C as controlled by a thermocoupled thermistor (Electr. lab, Copenhagen). In 6 expts. the tongue was quickly removed from the animal and placed in an organ bath containing a Krebs-glucose solution (see below). The bath was kept at 37°C and briskly bubbled with a gas mixture containing 4% CO₂ in oxygen.

B. Histological techniques

To visualize the vascular anatomy of the feline tongue the external carotid artery was cannulated in 4 expts. and a mixture (1:1) of filtered India ink and papaverine (1 mg/ml physiological saline) was injected. The injection was continued until the tongue attained a grayish colour which required an injection volume of 3–6 ml. After the injection the tongue was removed and the tissue was cleared by using the technique developed by Spaetholz (1888). After such treatment of the tissue the India ink filled blood vessels were easily seen in an ordinary microscope.

In 2 expts. parts of the cat's tongue were rapidly cut out and fixed in 10% neutral formalin. The blood vessels were prepared for routine paraffin embedding, cut at 4 µm, mounted and stained according to standard procedures.

C. Cryoscopic technique

While exposed to the solution under study the tongue was rapidly extirpated and immediately frozen in isopentane precooled by liquid nitrogen. The tongues were then sectioned in 15 µm thick slices in a cryostat at -25°C. The cutting was performed obliquely in relation to the long axis of the tongue's papilla so that one obtained cross-sections of papillae at various levels of their lengths (see Fig. 3). The sections were mounted on glass and examined in a room at -10°C with a microscope equipped with an aluminum block, the temperature of which was kept constant by a servosystem. It was increased in a step-wise fashion allowing a 4–10 min period for temperature equilibration between the metal and the tissue section. At each temperature the tissue section was photographed and the boundary between frozen and thawed tissue was estimated in relation to total papillary length. A tissue section was considered thawed when no ice crystals were observed in the section. The tissue cryostat was calibrated using tissue sections equilibrated with solutions of varying osmolalities as described in more detail by Jodal *et al.* (1978).

When increasing tissue temperature step-wise, it was possible to obtain 2–5 points relating tissue osmolality to total papillary length. These observations were treated by a computer in the following way (Jodal *et al.* 1978): to obtain the osmolality at the papillary tip, lines connecting the two top observations were extrapolated to the 5 per cent level of total papillary length. The upper 5 per cent were considered to be composed of epithelial cells. The base value (100 per cent papillary length) was set at 312 mOsm/kg H₂O, the mean value of cat plasma osmolality. With these assumptions the computer calculated the tissue osmolality at 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent of total papillary length. Moreover mean osmolalities in the tissue between 5 and 30 per cent and between 5 and 50 per cent of total papillary length as well as mean osmolality throughout the papillary tissue were determined.

TABLE I. Composition of the various solutions used in this study. Concentrations are expressed in mmol/l.

	Na ⁺	K	Mg ²⁺	Ca ²⁺	Choline	Cl ⁻	HCO ₃ ⁻	SO ₄ ²⁻	PO ₄ ³⁻	Glucose	Mannitol
art-glucose	147	4.7	1.2	2.5		134	25		1.2	30	
art-mannitol	147	4.7	1.2	2.5		134	25		1.2		30
art-glucose		4.7	1.2	2.5	147	134	25		1.2	30	
art-mannitol		4.7	1.2	2.5	147	134	25		1.2		30
art-glucose-mannitol	147	4.7	1.2	2.5			30	25	1.2	30	61

1. Saline

The physiological saline solution contained 0.9 g NaCl per liter water. Its osmolality measured 292 mmol/kg.
 (b) The composition of the other electrolyte solutions used are given in Table I.

2. Statistics

Statistical significance was tested using Wilcoxon nonparametric tests (Siegel 1956).

Results

(a) Histological observations

In the cat's tongue 3 different types of papillae were observed, i.e. filiform, fungiform and laminate. The present study was primarily devoted to the study of the vascular anatomy of the filiform papillae, and to some extent also to the fungiform ones.

1. *Filiform papillae* The filiform papillae were of different size and shape, and it was possible to differentiate between two main types in the cat according to the arrangement of the vessels, location on the tongue and extent of keratinization. (a) A small type with one or two vascular loops (Fig. 1). The intervascular distance measured about 50 µm, as estimated

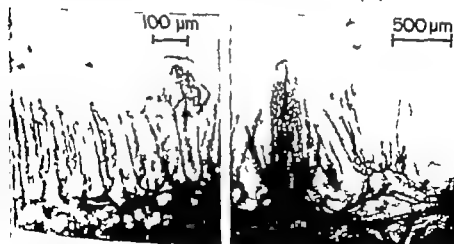


Fig. 1

Fig. 2

Fig. 1 The vasculature of papillae on the cat's tongue as demonstrated by an *in situ* injection of India ink. The right part of the Fig. 1 shows a fungiform papilla. The rest of the Fig. 1 illustrates the appearance of small filiform papillae. For further details, see text.

Fig. 2 The vascular architecture of large filiform papillae (left) and fungiform papillae (right). For further details, see text.

The papillae on the mammalian tongue seem to have a vascular arrangement resembling that of the intestinal villi at least to judge from studies performed on the dog (for review see Hellekant 1972). Thus, a countercurrent exchanger may exist also in these papillae. In the present study was initiated to test this hypothesis. Its purpose was twofold. First, it was deemed necessary to investigate the cat with regards to its vascular anatomy in the papillae of the tongue, since such studies are largely lacking. Second, experiments were performed using a cryoscopic technique, to establish the possible presence of a hyperosmolar region in the papillae, which would indicate the existence of a countercurrent exchanger.

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The tongue, placed in a little plastic cup *in situ* was exposed to an electrolyte solution of known composition by dripping the solution on the dorsum of the tongue for about 30 min at a rate of 1-2 ml/min. In most of the experiments the solution was heated to a temperature of 36-38°C as controlled by a thermocoupled thermometer (Electrolab, Copenhagen). In 6 expts. the tongue was quickly removed from the same and placed in a organ bath containing a Krebs-glucose solution (see below). The bath was kept at 37°C and briskly bubbled with a gas mixture containing 4% CO₂ in oxygen.

B. Histological techniques

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In 2 expts. parts of the cat's tongue were rapidly cut out and fixed in 10% neutral formalin. The blocks were prepared for routine paraffine embedding, cut at 4 µm, mounted and stained according to standard procedures.

C. Cryoscopic technique

When exposed to the solution under study the tongue was rapidly extirpated and immediately frozen in isopentane precooled by liquid nitrogen. The tongues were then sectioned in 15 µm thick slices in a cryostat at -25°C. The cutting was performed obliquely in relation to the long axis of the tongue's papillae so that one obtained crosssections of papillae at various levels of their lengths (see Fig. 3). The sections were mounted on glass and examined in a room at -10°C with a microscope equipped with an aluminum block, the temperature of which was kept constant by a servosystem. It was increased in a step-wise fashion allowing a 4-10 min period for temperature equilibration between the metal and the tissue section. At each temperature the tissue section was photographed and the boundary between frozen and thawed tissue was estimated in relation to total papillary length. A tissue section was considered thawed when no crystals were observed in the section. The tissue cryostat was calibrated using tissue sections equilibrated with solutions of varying osmolalities as described in more detail by Jodal *et al.* (1978).

When increasing tissue temperature step-wise, it was possible to obtain 5 points relating tissue osmolality to total papillary length. These observations were treated by computer in the following way (Jodal *et al.* 1978) to obtain the osmolality at the papillary tip. The line connecting the two top observations was extrapolated to the 5 per cent level of total papillary length. The upper 5 per cent were considered to be composed of epithelial cells. The base value (100 per cent papillary length) was set at 312 mOsm/kg H₂O the mean value of cat plasma osmolality. With these assumptions the computer calculated the tissue osmolality at 5, 10, 20, 30, 40, 50, 60, 70, 80 and 90 per cent of total papillary length. Moreover mean osmolalities in the tissue between 5 and 30 per cent and between 5 and 50 per cent of total papillary length as well as mean osmolality throughout the papillary tissue were determined.

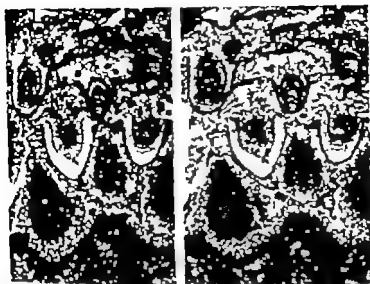


Fig. 1. *Left panel* Histological section of the tongue of cat photographed through microscope at temperature corresponding to freezing point depression of solution with an osmolality of 340 mOsm/kg H_2O . The tissue is sectioned so that cross sections of large filiform papillae are obtained. Ice crystals (arrows) close to the laminae (L, upper part of Fig.) are completely thawed. In the deeper part of the tissue (lower part of Fig.) ice crystals are seen. *Right panel* The same histological section as in left panel photographed at temperature corresponding to freezing point depression of solution with an osmolality of about 315 mOsm/kg H_2O . At this temperature the whole tissue is melted.

thawed. Hence, one may conclude that a gradient of tissue hyperosmolality exists, the highest osmolality being found at tips of the papillae. When the temperature of the tissue was increased to one corresponding to an osmolality of 310 mOsm/kg H_2O or less, the whole tissue including the solution on its surface, thawed.

Fig. 4 summarizes the results of 6 expts. in which the tongue has been exposed to isotonic solutions with varying amounts of glucose and sodium. The left panel illustrates the results on the large filiform papillae and the right panel the results on the small ones. Exposing the papillae to a Krebs-glucose solution produced a marked osmolar gradient along the length of the large papillae, the tip osmolality being on an average above 800 mOsm/kg H_2O (Table II). A similar gradient was also found in the small papillae although less pronounced. Substituting all sodium ions in the solution with choline (choline-glucose solution) actually decreased tissue osmolality in the large papillae while no significant effect was seen in the small filiform papillae (Fig. 4). Similarly removing glucose from the Krebs-glucose solution decreased tissue osmolality in the large papillae but not in the small ones (Fig. 5). Adding glucose from the sodium free solution, on the other hand, produced no significant effect on tissue osmolality in any type of filiform papillae (compare Fig. 4 and 5). In 6 expts. the chloride ions of the Krebs-glucose solution was substituted with sulphate keeping osmolality constant by addition of mannitol. This experimental procedure actually depressed tissue osmolality in the large papillae, while only a small, insignificant effect on the osmolality was observed in the small ones (Fig. 6).

TABLE II Tip and mean tissue osmolality in mOsm/kg H₂O in various portions of large filiform papillae of the cat's tongue during the different experimental conditions of this study. Mean values \pm S.E.

	Tip	From 5 to 30 of villous length	From 5 to 90 of villous length	Whole villous
Krebs-glucose (n = 12)	833 \pm 53	729 \pm 79	662 \pm 19	545 \pm 1
Krebs mannitol (n = 10)	594 \pm 3	528 \pm 15	489 \pm 11	464 \pm 11
Choline-glucose (n = 19)	414 \pm 13	391 \pm 10	374 \pm 9	347 \pm 6
Choline-mannitol (n = 12)	475 \pm 20	430 \pm 13	400 \pm 9	36 \pm 7
Krebs-glucose-sulphate (n = 16)	361 \pm 15	343 \pm 7	337 \pm 6	326 \pm 3
Ischaemia (n = 18)	414 \pm 11	395 \pm 9	377 \pm 7	351 \pm 5
NaCl 38°C (n = 4)	639 \pm 24	571 \pm 45	528 \pm 3	456 \pm 18
NaCl 20-22°C (n = 12)	448 \pm 57	387 \pm 76	373 \pm 20	352 \pm 12

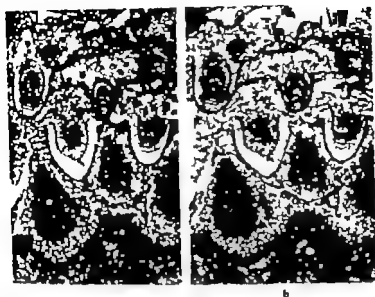
on the photographs taken of the histological specimens. These papillae were situated on tip and lateral sides of the tongue. The keratinization was scanty or none. 2 A larger conical or tusk-shaped type with a central vessel presumably an artery and a well developed capillary network (Fig. 2). This type was situated on the dorsum of the tongue, and the papillae were partly covered with a horny scale. The distance between the central vessel and the capillary network measured about 100 μ m. Within each of the two groups more simple as well as more complicated types occurred.

2. *Fungiform papillae* The fungiform papillae also differed in shape and vascular arrangement and two main types were discerned. 1 In the fungiform papillae on the tip and lateral sides of the tongue, the vascular supply consisted of a single vessel probably an arterial one. This vessel formed in the upper half of the papillae a few capillary loops (Fig. 1). 2 On the dorsum of the tongue the fungiform papillae were more heterogeneous in shape and vascular arrangement. In one type a ring of capillary loops were formed in the upper 2/3 of the papillae, the arterial and venous supply consisting of several vessels (Fig. 2). Other papillae were provided with a capillary network all the way from tip to base and a centrally running arterial vessel.

B. Cryoscopic findings

1. *Filiform papillae* The results of the cryoscopic investigations on the filiform papillae are given in Fig. 3-9 (osmolality gradients along the papillae) and in Tables II and I (osmolality in various parts of the papillae). The observations on the filiform papillae are described separately for small (type 1 as described above) and for the large papillae (type 2).

After exposing the dorsum of the cat tongue for 30 min to a Krebs-glucose solution, it was possible to demonstrate the presence of a hyperosmolar region at the tips of both the filiform and fungiform papillae. A representative experiment on the large filiform papillae is illustrated in Fig. 3. The left part of this figure shows the appearance of the frozen tissue sections at a temperature corresponding to 540 mOsm/kg H₂O. It can be seen that some cross sections of papillae in the upper part of the figure, i.e. in the tip portion of the papillae, have thawed. In the lower part of the figure black "grains" i.e. ice crystals, are abundant. The right part of the figure illustrates the appearance of the tongue papillae at a temperature corresponding to an osmolality of about 315 mOsm/kg H₂O. In this figure the whole tissue



b

Fig. 3. Left panel: Histological section of the tongue of a cat photographed through a microscope at a temperature corresponding to freezing point depression of a solution with an osmolality of 540 mOsm/kg H_2O . The tissue was sectioned so that cross sections of large filiform papillae are obtained. The cross sections (arrows) close to the lobes (L, upper part of Fig.) are completely thawed. In the deeper part of the tissue (lower part of Fig.) ice crystals are seen. Right panel: The same histological section as in (a) photographed at a temperature corresponding to freezing point depression of a solution with an osmolality of about 315 mOsm/kg H_2O . At this temperature the whole tissue is melted.

thawed. Hence, one may conclude that a gradient of tissue hyperosmolality exists, the highest osmolality being found at tips of the papillae. When the temperature of the tissue was increased to one corresponding to an osmolality of 310 mOsm/kg H_2O or less, the whole tissue including the solution on its surface, thawed.

Fig. 4 summarizes the results of 6 expts. in which the tongue has been exposed to isotonic solutions with varying amounts of glucose and sodium. The left panel illustrates the results on the large (filiform) papillae and the right panel the results on the small ones. Exposing the papillae to a Krebs-glucose solution produced a marked osmolar gradient along the length of the large papillae, the tip osmolality being on an average above 800 mOsm/kg H_2O (Table II). A similar gradient was also found in the small papillae although less pronounced.

Substituting all sodium ions in the solution with choline (choline-glucose solution) markedly decreased tissue osmolality in the large papillae while no significant effect was seen in the small filiform papillae (Fig. 4). Similarly removing glucose from the Krebs-glucose solution decreased tissue osmolality in the large papillae but not in the small ones (Fig. 5). Including glucose from the sodium free solution, on the other hand, produced no significant effect on tissue osmolality in any type of filiform papillae (compare Fig. 4 and 5).

In 6 expts. the chloride ions of the Krebs-glucose solution was substituted with sulphate and keeping osmolality constant by addition of mannitol. This experimental procedure markedly depressed tissue osmolality in the large papillae, while only a small, insignificant effect on the osmolality was observed in the small ones (Fig. 6).

TISSUE OSMOLALITY
mOsm/kg H₂O

1000

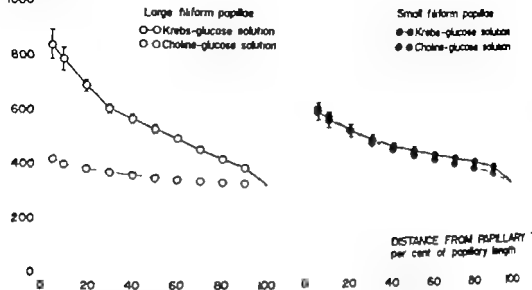


Fig. 4 Tissue osmolality in large and small tongue papillae when exposed in isotonic Krebs-glucose and choline-glucose solutions. The temperature of the solutions was maintained at 36–38°C. Bars denote S.E. At several points of the Fig. the S.E. is so small that it fits within the area of the filled circle. For number of observations, see Tables II and III.

Exposing the tongue to water markedly decreased osmolality in the small filiform papillae (Fig. 7), a decreasing osmolality from base to tip being observed. In the large papillae, on the other hand, no such marked hypoosmolality was apparent. In fact it was sometimes difficult to detect any decrease below isotonicity since the whole papilla thawed at a temperature just below the isotonic one. The general impression from these experiments was that exposing the large papillae to water decreased tissue osmolality to about 250 mOsm/kg H₂O at most. No clearcut gradient of the type illustrated on Fig. 7 could, however, be demonstrated.

In one series of expts. the tongue was exposed to an isotonic saline solution at two different temperatures. Fig. 8 illustrates the results obtained in the large papillae, which were

TABLE III Tip and mean tissue osmolality in mOsm/kg H₂O in various portions of small filiform papillae of the cat tongue during the different experimental conditions of this study. Mean values \pm S.E.

	Tip	From 5 to 30% of villous length	From 5 to 50% of villous length	Whole villous
Krebs-glucose (n=15)	589 \pm 30	528 \pm 22	497 \pm 17	476 \pm 13
Krebs-manitol (n=15)	568 \pm 21	528 \pm 17	502 \pm 15	490 \pm 13
Choline-glucose (n=18)	601 \pm 13	544 \pm 9	501 \pm 7	454 \pm 6
Choline-mannitol (n=13)	623 \pm 22	567 \pm 17	579 \pm 16	497 \pm 15
Krebs-glucose-sulphate (n=17)	528 \pm 20	480 \pm 17	450 \pm 14	399 \pm 9
Jachemala (n=18)	480 \pm 8	452 \pm 6	431 \pm 5	390 \pm 5
Water (n=18)	116 \pm 6	170 \pm 7	195 \pm 6	241 \pm 5

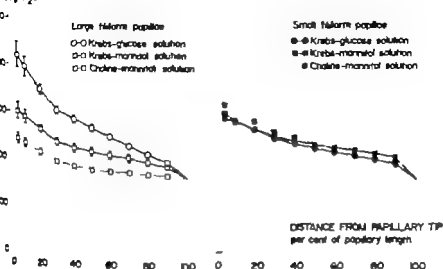
Tissue Osmolality
mOsm/kg H₂O

Fig. 1. Tissue osmolality in large and small filiform papillae of the cat tongue recorded *in vivo* exposed to Krebs-glucose, Krebs-mannitol or choline-mannitol solutions. For further details of the figure, consult legend to Fig. 4.

only ones studied in this series of expts. The lowering of temperature decreased tissue osmolality significantly.

Finally in 6 expts. the tongue was removed and placed in a well oxygenated organ bath containing Krebs-glucose. The results obtained are illustrated in Fig. 9 (ischemia). A significantly decreased tissue osmolality was apparent in both types of papillae although the depression was most marked in the large ones.

2. Fungiform papillae Due to their shape and limited number it was not possible to cut the fungiform papillae in cross sections in the way shown in Fig. 3 for the filiform ones. For such reasons it was difficult to determine the precise localisation of an observed cross-section along the length of a papilla. However it was quite clear that a hyperosmolar region was also present in the fungiform papilla. In the series of expts. with a Krebs-glucose solution, an attempt was made to estimate the tip osmolality. It was shown to be at least 500 mOsm/kg H₂O in the small type of fungiform papillae and at least 600 mOsm/kg H₂O in the large ones.

Discussion

In this study both anatomical and physiological observations have been reported, suggesting the presence of a countercurrent exchanger in the filiform and fungiform papillae of the cat tongue. In an anatomical investigation it was demonstrated that hairpin vascular loops are present in both types of papillae. The appearance of the vascular architecture observed in this study was the same as that earlier described for the papillae in dog and man (Hellekant

TISSUE OSMOLALITY
mOsm/kg H_2O

1000

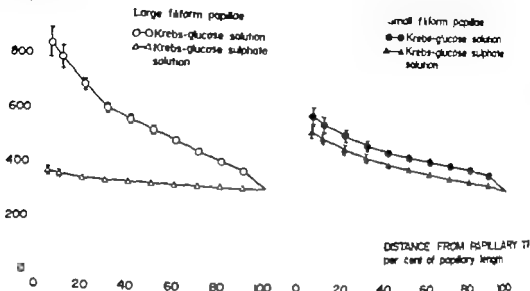


Fig. 6. Tissue osmolality in large and small filiform papillae when exposed to Krebs-glucose or Krebs-glucose-sulphate solutions, illustrated in the same way as in Fig. 4.

1972). The intervascular distance between the two limbs of the vascular loops could be measured in the filiform papillae. In the small type it was estimated to 50 μm and in the large type to about 100 μm . A substance with a free diffusion coefficient in water of $10^{-5} cm^2/s$ would reach a 50% diffusion equilibrium across the mentioned distances in 0.1–0.2 s, respectively. The diffusion process is, hence, fairly rapid across the measured intervascular distance. However, the possible functional effects of the vascular arrangement in the papillae do not solely depend on time for diffusion but also on such hemodynamic parameters as e.g. mean transit time in the vascular loops. No data are at present available on the detailed hemodynamics of the papillae of the tongue.

The possible functional implications of the vascular arrangement was investigated by determining tissue osmolality with a cryoscopic technique originally developed for the small intestine (Jodal *et al.* 1978). The tongue was exposed to different electrolyte solutions and marked hyperosmolality was observed at the papillary tip when exposing the tongue to solutions containing both sodium and glucose, two molecules known to interact and facilitate their mutual transcellular transport (Crane 1968). An osmolality gradient was observed toward the base of the papillae. Furthermore, performing experiments in the absence of blood flow markedly decreased the measured tissue osmolality, particularly in the large filiform papillae (Fig. 9).

These observations are fully consistent with the presence of a countercurrent multiplier in the papillae of the tongue. Fig. 10 depicts the proposed mechanisms in the large and small filiform papillae. The active transport of a solute increases its plasma concentration in the subepithelial capillary network at each point along the length of the papillae. In the large papilla such an active transport would produce a higher concentration of the solute in the

Tissue Osmolality
mOsm/kg H₂O

1000

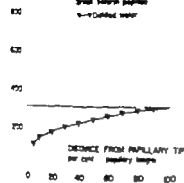
Small filiform papillae
→ Distilled water

Fig. 7

Tissue Osmolality
mOsm/kg H₂O

1000

100 mmoles NaCl/l, 37°C

100 mmoles NaCl/l, 20-22°C

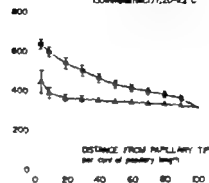


Fig. 8

Fig. 7. Tissue osmolality in the small filiform papillae when the tongue was exposed to distilled water. Same as the same as in Fig. 4.

Fig. 8. Tissue osmolality in the large filiform papillae when the tongue was flushed with an isotonic sodium chloride solution at two different temperatures. For further details of the figure, consult legend to Fig. 4.

capillaries than in the central arterial vessel and the solute may diffuse along this concentration gradient to be brought back towards the tip by the central vessel. If the solute capillary permeability is low water may move in the opposite direction driven by the osmotic forces caused by the active transport mechanism. In the small filiform papillae with only one or a few capillary loops the concentration of the actively transported solute is higher in the trailing limb of the loop than in the supplying one (right panel of Fig. 10). As in the large filiform papillae the solute may move along its concentration difference and/or water may be transported secondary to osmotic forces.

The described mechanisms produce the same end result, *i.e.* an increasing tissue hyperosmolality from base to tip in the papillae as reported in this study. The results clearly suggest that the countercurrent multiplier is most efficient in the large filiform papillae as compared to the small ones. This difference may be ascribed to differences in the solute transport capacity or to the obvious differences in vascular anatomy (Fig. 1 and 2).

It might be argued that the demonstration of a hyperosmolar region in the papillary tip and a decreasing osmolar gradient along the papilla may be attributable to the presence of active pumps with different transport capacities. If so blood flow would tend to decrease the osmolality by carrying away some of the absorbed solute, and one would find a higher tissue osmolality during *in vitro* conditions than *in vivo*. As shown in Fig. 9 the tissue osmolality was actually decreased *in vitro* particularly in the large papillae which argues against the view that the observed hyperosmolar compartment was solely due to the distribution of this pump.

An attempt was made in this study to elucidate which solute that established the tissue hyperosmolality. For such reasons the compositions of the solutions were varied particu-

TISSUE OSMOLALITY mOsm/kg H_2O

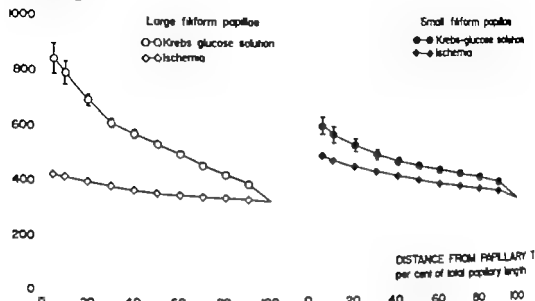


Fig. 9. Tissue osmolality in large and small filiform papillae when exposing the tongue to a Krebs-glucose solution *in vivo* (circles) or *in vitro* (squares). For further details of the figure, consult legend to Fig. 4.

larly with respect to their contents of sodium, chloride and glucose. In the large filiform papillae the exchange of sodium with choline or glucose with mannitol markedly decreased the observed papillary hyperosmolality (Fig. 4 and 5). These observations suggest that active transport of sodium is of prime importance for establishing the hyperosmolality the same way as has been shown in the cat intestine (Haljamäe *et al.* 1973, Jodal *et al.* 1977). Hence, the epithelium on the large filiform papillae seems to be provided with polarized cells that seem to be capable of moving sodium against a concentration gradient.

Exposing the large papillae to a solution in which the chloride ions had been substituted with sulphate ions also markedly lowered the tissue osmolality as compared to the one at during exposure to a Krebs-glucose solution. Sulphate ions, like most divalent ions, are poorly transported across most epithelia (Wilson 1962), probably including also the epithelium of the tongue.

LARGE FILIFORM PAPILLA SMALL FILIFORM PAPILLA

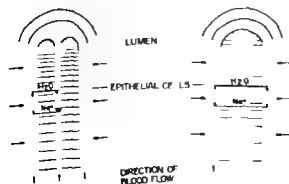


Fig. 10. The proposed current multiplication mechanism in large and small filiform papillae schematically illustrated. The increased osmolality in tissue is indicated by an increased number of horizontal lines per unit length. For further details of the figure, see text.

lin of the tongue. If so, the inability of the sulphate ions to move across the epithelium may secondarily lead to a low transport rate of sodium explaining the observed low tissue osmolality. Alternatively all the results in this study obtained with solutions of varying composition may be explained by the presence of an active chloride transport mechanism.

It might be argued that the presence of a hornpick on the large filiform papillae would provide such a hindrance to solute transport that Na^+ glucose and sodium would not reach the epithelium. However the hornpick only covers part of the papilla. Furthermore, the nasal epithelium of the rat has been shown to have a measurable permeability to glucose (Narita 1971). Finally the frog skin with a histological appearance similar to that of the mammalian tongue is provided with active transport systems.

The results obtained on the small filiform papillae differed markedly from that described above for the large ones. The osmolar gradient found in the small papillae was largely unaffected by variations in sodium and glucose contents of the applied solutions (Fig. 4 and 5). These observations suggest that no active transport of sodium was involved in the establishment of the tissue hyperosmolality. The Krebs-glucose solution containing sulphate did not significantly decrease the gradient. This observation seems to suggest that the chloride ion is not involved in producing the tissue osmolality and that other transport mechanisms must be responsible. Furthermore, the water permeability of the small papillae was greater than that of the large ones to judge of the marked hypotonicity found in the small but not in the large papillae (Fig. 7).

The analysis of the fungiform papillae could for technical reason not be carried out in the detailed way that is described for the filiform papillae above. This was disappointing since the fungiform type, provided with taste buds, seems from a functional point of view more interesting than the filiform papillae. It was, however, clearly demonstrated that a hyperosmolar compartment also existed in the tissue of these papillae.

The physiological significance of the proposed countercurrent multiplier in the tongue is not known. With the hyperosmolality present in the papillae it seems probable that the tongue absorbs water along the transepithelial osmotic gradient. The forces involved may be very great since 1 osm corresponds to a pressure of about 20 mmHg. Assuming a reflection coefficient of 1 the transepithelial osmolality difference across the papillary tip (around 900 mosm/kg H_2O) corresponds to a pressure difference of 18 000 mmHg. Although such forces should produce a fast absorption of water it must be quantitatively of minor importance compared to the absorptive capacity of the small bowel due to the difference in absorptive area.

The fungiform papillae are supplied with taste buds and the countercurrent exchange may hence, possibly be important for taste. One may only speculate on this and two tentative proposals will be made. First, it has been demonstrated in the small intestine that its exchanger delays the net blood absorption of lipophilic solutes due to their trapping in the exchanger (Savitsky 1973; Jodal *et al.* 1977). A comparatively high concentration of the lipid soluble compound is then built up in the tissue due to its "recirculation" in the villi. In a similar way one may envisage a trapping of lipid soluble "taste" compounds in the exchanger delaying their net blood transport. Such a mechanism would maintain a high tissue concentration of the compound for a longer time than in the absence of an exchanger.

Second, it may be inferred from the results of this study that the extracellular concentration of electrolytes, including potassium, may be much higher than normal at the papillar tips. Such high electrolyte concentrations may be of importance for the activation of the taste buds and possibly also of the free nerve endings in the filiform papillae.

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References

- BOND J H D J LEVITT and M D LEVITT Quantitation of countercurrent exchange during passive absorption from the dog small intestine—evidence for marked species differences in the efficiency of exchange. *J clin Invest* 1977 59 308–318.
- CRANT R K Absorption of sugars. In *Handbook of Physiology* 1968. Section 6. Vol. III 1323–1351.
- HALLBÄCK, D A L, M JODAL and O LUNDGREN, Countercurrent multiplication of sodium in intestinal villi during absorption of sodium chloride. *Acta physiol. scand.* 1973. 89 580–593.
- HALLBÄCK, D A L, HULTÉN M JODAL, J LINDBÄCK and O LUNDGREN, Evidence for the existence of countercurrent exchanger in the small intestine in man. *Gastroenterology* 1978 a. 74 683–690.
- HALLBÄCK, D A L, M JODAL and O LUNDGREN, Tissue osmolality in the papillae of the cat's tongue. *Acta physiol. scand.* 1978 b. 102 32 A.
- HELLEKANT G Circulation of the tongue. In *Oral Physiology* (Eds. Nils Emmelin and Yngve Zetterstrom) pp. 127–137 1972. Pergamon Press Ltd., Oxford.
- JODAL, M D A, HALLBÄCK and O LUNDGREN, Tissue osmolality in intestinal villi during intestinal perfusion with isotonic electrolyte solutions. *Acta physiol. scand.* 1978. 102. 94–107.
- JODAL, M J SVANVİK and O LUNDGREN, The importance of the intestinal countercurrent exchanger for ^{86}K absorption from the feline gut. *Acta physiol. scand.* 1977 100. 412–423.
- LUNDGREN, O Studies on blood flow distribution and countercurrent exchange in the small intestine. *Acta physiol. scand.* 1967 Suppl. 303.
- LUNDGREN, O The circulation of the small bowel mucosa. *Gut* 1974. 15 1005–1013.
- MISTRETTA, C M Permeability of tongue epithelium and its relation to taste. *Amer J Physiol.* 1971 220 116–1167.
- SELIG, S., *Nonparametric statistics for the behavioral sciences*. McGraw-Hill Book Company Inc. Tokyo 1956.
- SPALTENHOLZ, W Die Verteilung der Blutgefäße im Muskel. *Abhandl. Säcker. Ges. Natur. Phys.-Med.* 1838. 14 309–328.
- SVANVİK, J Mucosal blood circulation and its influence on passive absorption in the small intestine. A experimental study in the cat. *Acta physiol. scand* 1973 Suppl. 385.
- WILSON, T H *Intestinal absorption*. W B. Saunders Co. Philadelphia, London 1962.

Plasma catecholamines, cyclic AMP and metabolic substrates in hemorrhagic shock of the rat. The effect of adrenal demedullation and 6-OH-dopamine treatment

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Abstract

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Plasma catecholamines, cyclic AMP and metabolic substrates in hemorrhagic shock of rats was studied in 4 groups of animals: 1) Control rats, 2) rats with adrenal demedullation, 3) rats with 6-OH-dopamine induced chemical sympathectomy and 4) rats with combined demedullation and sympathectomy. The rats were bled to systemic blood pressures of 35 mmHg.

The basal plasma level of noradrenaline (NA), adrenaline (A) and dopamine (DA) in control animals was about 1 nmol/l. After hemorrhage for 1 h the A levels had reached 50 nmol/l and there was little further rise after 4 h. The rise was characterized by demedullation but unaffected by sympathectomy. NA levels rose continuously in the control and in the sympathectomized rats. At 1 h the level was about 4 nmol/l and 4 h about 20 nmol/l. The demedullated rats showed 3-fold increase in circulating NA, while little or no change was seen in the combined demedullated and sympathectomized rats. DA levels did not change in any of the groups during the first hour, but were markedly elevated after 4 h of hypotension.

Cyclic AMP and glucose levels in plasma showed rapid increase 1 h after hemorrhage and thereafter returned to or below basal values. Demedullation largely prevented the increase, while sympathectomy had no effect. The increase in lactate and pyruvate levels were diminished but not characterized by either sympathectomy or demedullation. Glyceral levels were unchanged and FFA decreased in all groups of rats.

The results show that the adrenal medulla is the dominating source of plasma catecholamines in hemorrhagic shock in rats. The initial increase in plasma glucose and cyclic AMP appear to be largely mediated by adrenal A. The subsequent fall in these parameters and derangement of circulatory homeostasis are not the present shock model, primarily due to failure of catecholamine secretion, but rather to decreased sympathetic outflow to peripheral tissues to catecholamine stimulation.

It is well known that the sympatho-adrenal system is activated by hemorrhage (see Chien 1977). Thus, there are several reports that in dogs bled to 40 mmHg plasma adrenaline (A) levels increase 50-fold and plasma noradrenaline (NA) levels about 10-fold (see Watts 1965). There is evidence that a major source of plasma catecholamines in this condition is the

adrenal medulla (Watts 1965). Recent advances in the methodology of catecholamine assay in plasma have, however, indicated that most, if not all, the older determinations are in error. Moreover, most of the data on catecholamine levels in shock are obtained in larger animals such as cat and dog, while much of the data concerning survival etc. are obtained on small rodents such as the rat.

We have studied the effect of adrenal demedullation and/or chemical sympathectomy on the metabolic responses to bleeding in a previously characterized modified Wiggles shock model in the rat (Whitgham and Weil 1966, Farnebo and Hamberger 1977, Farnebo *et al.* 1977). A new sensitive and specific method for the determination of plasma A, N, and dopamine (DA) levels was used (Keller *et al.* 1976, Mefford *et al.* 1977, Hallgren *et al.* 1978) to study the induced changes in plasma catecholamine levels.

Several metabolic alterations result from hemorrhage. There is an increase in hepatic glycogenolysis and glucose output (see Levenson *et al.* 1961), which leads to elevated plasma glucose levels (see Järhult 1975). This response is most commonly attributed to A release from the adrenals (*e.g.* Engel 1943, Halmagyi *et al.* 1967, Hiebert *et al.* 1973), but Järhult (1975) suggested that activation of sympathetic nerves to the liver was at least as important a mechanism. Plasma cyclic AMP also increases during bleeding in dog (McArdle *et al.* 1974) and rat (Farnebo *et al.* 1977). It is not known to what extent sympathetic nerves and the adrenal medulla contribute to the elevation of plasma cyclic AMP following hemorrhage. Bleeding also induces several other metabolic alterations such as increased lactate and pyruvate levels as well as changes in fat metabolism (*e.g.* Weil and Afifi 1970, Kovach *et al.* 1970, Farnebo *et al.* 1977). The role of circulating and/or locally released catecholamines in the induction of these metabolic events is similarly unknown. A second aim of the present study has therefore been to study the metabolic changes induced by hemorrhage simultaneously with the determination of catecholamines.

Material and Methods

Males Sprague-Dawley rats weighing about 250 g were used. They were given water and food standard diet which was withheld about 18 h before the experiment. Four experimental groups of rats were used. 1. *Untreated controls*. 2. *Demedullated rat*. About 3 weeks before the hemorrhage experiment the rats were anesthetized with sodium pentobarbital 60 mg/kg i.m. A midline skin incision was made on the back through which both adrenals could be removed. The cortex and medulla were separated under microscope and numerous small pieces of the cortex transplanted under the renal capsule (Banno *et al.* 1970). The rats survived for 3 weeks postoperatively and their weight gain on the standard diet was the same as that of the controls. Since animals with deficiency of adrenal cortical function rapidly lose weight and die within weeks (Hahn 1969) this suggests that the adrenal cortical transplants were functioning. When the rats were killed, it was found that much of the cortical material had been revascularized in agreement with the results from the original description of the transplantation method (Banno *et al.* 1970), which paper also reports of corticosteroid assays are given. Numerous smears of the transplants were made to try to localize any remaining medulla using fluorescence histochemistry but no such tissue could be seen. 3. *Chemically sympathectomized rat*. The rats were injected with 6-OH DA 50 mg/kg i.v. 3–5 days before the hemorrhage experiment. When these rats were killed whole mounts of iris were examined in the fluorescence microscope to control the degree of sympathectomy. Only one to three terminal axons could be seen in each iris which demonstrates a high degree of denervation after 6-OH DA at least in this tissue. 4. *Demedullated and sympathectomized rat*. Both the procedures described under 2 and 3 were performed.

Experimental procedures

The experimental haemorrhage model used is principally that described by Whigham and Wal (1966) with minor modifications (Farber and Hershberger 1977). The rats were anaesthetized with sodium pentobarbital 50 mg/kg. Cannulized catheters were inserted in both femoral arteries and heparin 300 U/kg was injected. The right catheter was connected to Statham transducer and the blood pressure continuously recorded on Grass polygraph. After rest period of 30 min the left femoral catheter was connected to a plastic heparinized syringe connected to mercury manometer which was adjusted to 35 mmHg. Blood was then allowed to flow out into the reservoir. The blood pressure, pulse rate and shed blood volume were continuously recorded. After 15 min the blood pressure was 40-45 mmHg in all groups of rats, and after 45 min the arterial blood pressure had reached 35 mmHg. Samples of 2 ml blood were taken for analysis of catecholamines and metabolites by disconnecting the femoral catheter to the reservoir and allowing the blood to drip into ice-chilled tubes. In the rats subjected to haemorrhage samples were taken at the beginning of bleeding and after the scheduled time of bleeding. In the non-bled controls, blood was taken only once.

Determination of catecholamines

For the estimation of the blood, plasma samples of 1 ml were taken for analysis. After addition of metabisulphite as internal standard and addition of sodium metapermanganate to prevent oxidation the catecholamines were adsorbed on 15 mg of alumina (BDH, Poole, England). After washing, the eluate solution was dried with 50 µl PCA, 20 µl of each was injected into high performance liquid chromatograph equipped with an electrochemical detector. This method has been developed by Adams and co-workers (Keller *et al.* 1976, McFarrell *et al.* 1977) and applied to plasma (Haffner *et al.* 1978). For detailed description and discussion of the method the reader is referred to the latter paper.

Determination of cyclic AMP

Cyclic AMP is generally determined directly on separated plasma samples collected in EDTA (final concentration 10 mM) essentially as described by Brown *et al.* (1972). Plasma samples of 50 µl were incubated with 250 µl (or 10 µl in 250 µl) 40 mM Tris-Cl and 7 mM β-mercaptoethanol, 5 mM EDTA and 42 M NaCl containing 3H-cyclic AMP (1 pmol) and sufficient amount of binding protein to ensure 30-80% binding of the labelled cyclic AMP. Purification of the plasma cyclic AMP by perchloric acid deproteinization followed by cation or anion (AG 1 8 or AG 50 8) exchange chromatography gave identical values to separated samples when corrections for losses are made. Treatment with phosphodiesterase (usually purified from rat brain) removed 90-95% of the apparent cyclic AMP activity. Serial dilutions of the plasma left on the standard curve. The authenticity of the cyclic AMP was therefore considered established.

Assay of glucose and organic substrates

Glucose was measured by glucose oxidase method (Gloss), glycerol enzymatically (Larrell and Tibbings 1967), l-lactate enzymatically (Larrell and Tibbings 1967), on plasma. Lactate and pyruvate in samples of 1 ml were precipitated with 0.4 M perchloric acid as determined enzymatically (TCB, TCC, Boehringer).

Separation of cyclic AMP from other nucleotides and bases

In the experiments where the fate of 3H-cyclic AMP was studied, plasma and tissues (liver, kidney) were homogenized in perchloric acid (final concentration 0.4 M). After standing in the cold for 30 min the samples were centrifuged and the supernatant neutralized with 4 M KOH and 1 M Tris-Cl. An aliquot was treated with equal volumes of 0.4 M ZnSO₄ and 0.4 M Na₂CO₃ which precipitates all nucleotides except cyclic AMP (Chen and Lin 1974). The radioactivity in the precipitate and supernatant was then counted by liquid scintillation. An aliquot of this supernatant or of the original supernatant was chromatographed on short (0.7-1.5 cm) AG 1 8 (200-400 mesh, Cl) columns. Fractions were eluted by 0.1 M Tris-Cl pH 7.4 and nucleotides by 2 M HCl. The radioactivity was then counted. 3H-cyclic AMP, other nucleotides, nucleosides and bases.

Drugs:
3H-Adenosine 3',5'-cyclic phosphate as the monoisotopic salt (26 Ci, mmol) was obtained from the Radiochemical Centre, Amersham, England, unlabelled cyclic AMP, AMP, ADP, ATP and adenosine from Boehringer, Mannheim, FRG. The cyclic AMP binding protein was prepared as described by Brown *et al.*

TABLE 1 The effect of demedullation (demed) chemical sympathectomy (6-OH DA) and of the combined treatment on arterial blood pressure and on arterial catecholamine levels. Results are given means \pm S.E. Number of observations is given within parenthesis.

Treatment		BP _{II} (mmHG)	A (nM)	NA (nM)	DA (nM)
Control	0 h	106 \pm 4 (20)	0.92 \pm 0.23 (12)	1.16 \pm 0.14 (12)	1.08 \pm 0.26 (12)
	4 h		3.47 \pm 1.42 (3)	2.07 \pm 0.31 (3)	2.77 \pm 0.14 (3)
Demed	0 h	97 \pm 3 (20)	ND ^a (11)	0.70 \pm 0.07 (11)	TA ^b (11)
6-OH DA	0 h	93 \pm 3 (18)	0.71 \pm 0.21 (10)	0.68 \pm 0.46 (10)	TA (10)
Demed + 6-OH DA	0 h	90 \pm 2 (2)	TA ^b (10)	0.4 \pm 0.07 (10)	TA ^b (10)

^a Not detectable.

^b Trace amounts found in 1-2 samples.

1972. Nont GSX was obtained from N.V. Algemeene Nont, Amersfoort, Holland, and reagents for the assay of lactate and pyruvate from Boehringer Mannheim, FRG and for the assay of glucose from Ket Stockholm, Sweden. Adrenaline, noradrenaline and dopamine were obtained as hydrochlorides from Sigma, St. Louis, USA. 6-hydroxy-dopamine was kindly supplied by AB Astra, Södertälje, Sweden, and begun by AB Vitrum, Stockholm, Sweden. AG 1-8 and AG 50-8 ion-exchange resins (Bio Rad, Richmond, USA) were extensively washed before use. Other chemicals were reagent grade from ordinary commercial suppliers.

Results

Effect of demedullation and sympathectomy

The effect of demedullation and sympathectomy on mean arterial blood pressure and on arterial plasma catecholamine levels is shown in Table I. The blood pressure before bleeding was significantly reduced in all groups of treated animals compared to the controls ($p < 0.01$). Sympathectomy appeared to have a larger effect on blood pressure than demedullation.

In control animals arterial A, NA and DA levels were approximately equal (Table I). In animals kept anesthetized for 4 h but not subjected to bleeding there was an approximately 3-fold increase in the level of each catecholamine. Following demedullation plasma A was undetectable, while 6-OH DA treatment had no significant effect on A. The plasma NA levels were reduced by some 40% by either demedullation or sympathectomy and by 65% by the combined treatment. Plasma DA was reduced to trace concentrations by either treatment.

The basal levels of plasma cyclic AMP, glucose, lactate, pyruvate, glycerol, and FFA in the 4 groups of animals are given in Table II. Although there were several statistically significant differences between the basal levels in the treated and untreated rats the absolute magnitude was generally small and the physiological significance is unclear. In the control rats glucose, lactate, pyruvate and FFA were increased by just keeping the animals for 4 h.

	Control		Dexam		6-OH-DA		Dexam 6-OH-DA	
	0	4 h	0	4 h	0	4 h	0	4 h
Cycle AMP nmol/l	15.1 ± 2.0 (23)	14.0 ± 2.0 (5)	30.5 ± 7.0 ^a (22)	12.7 ± 7.8 (5)	28.1 ± 6.0 ^a (8)	26.8 ± 10.0 ^b (4)	10.8 ± 2.3 ^a (12)	8.2 ± 2.7 ^a (5)
Glucose nmol/l	—	8.77 ± 0.24 ^{***} (7)	6.23 ± 0.41 (25)	9.20 ± 0.17 ^a (4)	5.05 ± 0.40 ^a (8)	6.25 ± 0.40 ^{***} (6)	7.39 ± 0.14 ^a (11)	7.54 ± 0.24 ^a (5)
Lactate nmol/l	6.94 ± 0.04 (44)	1.19 ± 0.2 ^a (8)	1.32 ± 0.15 ^a (25)	1.80 ± 0.24 ^a (6)	0.92 ± 0.09 (8)	0.91 ± 0.04 (4)	0.73 ± 0.07 ^a (12)	0.56 ± 0.04 (5)
Pyruvate nmol/l	6.5 ± 4 (35)	1.17 ± 0.6 (5)	6.7 ± 8 (25)	9.9 ± 1.6 (6)	5.8 ± 1.2 (8)	5.4 ± 3.0 (4)	8.0 ± 5 ^a (11)	7.5 ± 4 (4)
Glycerol nmol/l	0.20 ± 0.01 (41)	0.22 ± 0.06 (7)	0.17 ± 0.01 (25)	0.18 ± 0.07 ^a (4)	0.13 ± 0.01 ^a (8)	0.15 ± 0.003 ^b (4)	0.70 ± 0.01 (12)	0.30 ± 0.02 ^{ab} (5)
FFA nmol/l	1.04 ± 0.20 (16)	1.44 ± 0.33 (4)	0.75 ± 0.00 ^a (25)	0.79 ± 0.13 ^a (5)	1.24 ± 0.11 ^a (8)	1.87 ± 0.49 ^{***} (4)	—	—

Significantly different from compounds 8 control (p = 0.05).

Significantly different from corresponding control (p < 0.01).

Significantly different from corresponding control (p < 0.001).

Significantly different from 0-value value ($p < 0.05$)Significantly different from 0-time values ($p < 0.01$).^a Significantly different from 0-time value ($p < 0.001$)

TABLE I The effect of demedullation (demed) chemical sympathectomy (6-OH-DA) and of the combined treatment on arterial blood pressure and on arterial catecholamine levels. Results are given as means \pm S.E. Number of observations is given within parenthesis.

Treatment		BP _{II} (mmHg)	A (nM)	NA (nM)	DA (nM)
Control	0 h	106 \pm 4 (70)	0.9 \pm 0.23 (12)	1.16 \pm 0.14 (12)	1.08 \pm 0.36 (12)
	4 h		3.47 \pm 1.4 (3)	2.07 \pm 0.51 (3)	2.77 \pm 0.84 (3)
Demed	0 h	97 \pm 3 (20)	ND ^a (11)	0.70 \pm 0.07 (11)	TA ^b (11)
6-OH DA	0 h	93 \pm 3 (18)	0.71 \pm 0.21 (10)	0.68 \pm 0.246 (10)	TA (10)
Demed + 6-OH DA	0 h	90 \pm (22)	TA ^b (10)	0.42 \pm 0.07 (10)	TA ^b (10)

^a Not detectable

^b Trace amount is found in 1 sample.

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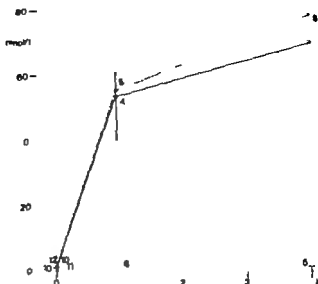


Fig. 2. Effect of bleeding on plasma adrenaline. \circ — \circ Control, \square — \square Demedullated, \circ — \circ Sympathectomized, \square — \square Demedullated and sympathectomized. Mean \pm S.E. Number of observations are given.

The NA levels rose about 3-fold 1 h after bleeding. This early rise was not altered by either demedullation or by sympathectomy alone (Fig. 3), but prevented by combined demedullation and sympathectomy. In intact rats there was a further 5-fold increase in NA levels between 1 and 4 h after bleeding. Interestingly this secondary rise in NA levels was prevented by demedullation, but not by sympathectomy.

The changes in DA levels following bleeding are illustrated in Fig. 4. There was no change in plasma DA during the first hour of hypotension. On the other hand, between 1 and 4 h after bleeding there was approximately a 20-fold increase, which was prevented by neither demedullation nor sympathectomy alone, but significantly reduced by the combination.

Heart catecholamines

The effects of 6-OH-DA-treatment, adrenal demedullation and bleeding on heart tissue catecholamine levels are presented in Table III. In control animals there was a significant increase in tissue A levels following bleeding, but no significant changes in NA and DA levels. Following demedullation no A could be detected in the heart. In these animals there was a significant ($p < 0.05$) fall in tissue NA content and rise in tissue DA content between 1 and 4 h following bleeding. In the sympathectomized rats the levels of all 3 catecholamines were reduced but a small rise in tissue A levels during the hypotensive period could still be observed.

Plasma cyclic AMP

There was a highly significant increase in plasma cyclic AMP levels in all groups of animals following bleeding (Fig. 5a). The increase in cyclic AMP after 1 h was not significantly altered by 6-OH-DA treatment, but was significantly reduced by demedullation and still further reduced by combined demedullation and sympathectomy. After 1 h the levels of cyclic AMP tended to fall. In the intact and sympathectomized animals the levels were

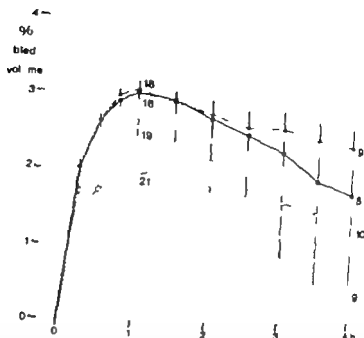


Fig 1 Bled volume. Rats were connected to a reservoir with a pressure of 35 mmHg. Bled volume is expressed as per cent of body weight. ●-● Control. ○-○ Demedullated. □-□ Sympathectomized. Mean \pm SE. Number of animals at 1 and 4 h are given. In the demedullated group some rats took back both their whole bled volume as well as saline from the reservoir.

The same pattern was not seen in the treated rats and may therefore be related to the increase in plasma catecholamines that occurred during the 4-h period in the control group but was not present in the treated animals.

Blood volume loss and heart rate. When the animals were allowed to bleed into the reservoir against a pressure of 35 mmHg, there was an initial rapid blood loss so that between 1 and 2% of the body weight was lost in 15 min. Thereafter bleeding proceeded at a lower rate for about 45 min (Fig. 1). The maximal amount of blood lost was unchanged by sympathectomy but reduced by about 20% by demedullation and by about 30% by combined demedullation and sympathectomy. After about 1 h of hypotension the animals started to take back fluid from the reservoir. This decompensation phase was reduced by sympathectomy whether the rats were demedullated or not.

In all groups of animals there was a transient bradycardia. It was maximal 5-10 min after the start of bleeding and thereafter the heart rate returned towards control. The reduction of heart rate was unchanged by adrenal demedullation, but significantly reduced by 6-OH DA treatment.

Plasma catecholamines

Following 1 h of hypotension the plasma A levels had increased to about 50 nmol/l. A further increase to 70 nmol/l occurred after 4 h of hypotension (Fig. 2). The A response was unaltered by chemical sympathectomy but completely eliminated by adrenal demedullation (Fig. 2).

Let II. Osteoblasts levels in heart subjected to hemorrhage. The animals were untreated (control), demedullated (den), chemically sympathectomized (6-OH-DA) or demedullated and sympathectomized (den+6-OH-DA). An unbled control was also included. Mean \pm SE. Number of experiments in brackets. The values are expressed as pmol/kg.

Ex	Animal	Control		Den	6-OH-DA	Den+6-OH-DA
		Not bled	Bled			
III	Nonadrenals		6.61 ± 0.20 (4)	7.08 ± 0.30 (6)	0.55 ± 0.05 (5)	0.49 ± 0.01 (4)
	Adrenals		0.85 ± 0.17 (4)	ND (6)	0.09 ± 0.01 (5)	ND (4)
	Deposits		0.05 ± 0.01 (4)	0.07 ± 0.01 (6)	0.06 ± 0.04 (5)	0.02 ± 0.01 (4)
IV	Nonadrenals	6.51 ± 0.51 (4)	5.58 ± 0.32 (4)	4.87 ± 0.84 (4)	0.52 ± 0.04 (5)	0.53 ± 0.03 (5)
	Adrenals	0.20 ± 0.01 (4)	2.96 ± 1.12 (4)	ND (4)	0.23 ± 0.01 (5)	ND (5)
	Deposits	0.32 ± 0.13 (4)	0.05 ± 0.01 (4)	0.32 ± 0.06 (4)	ND (5)	0.03 ± 0.01 (5)

ND not detectable.

of cyclic nucleotide phosphodiesterase in plasma of rats (*cf* Broadus 1977). The content of nucleotides in kidney and liver of the shock animals was lower than that of the intact animals. Thus, in kidney there was radioactivity corresponding to 1.73 ± 0.08 nmol/g in the control group and 1.27 ± 0.08 nmol/g in the shock group ($n=3$). In the liver the corresponding figures were 0.39–0.05 and 0.23 ± 0.02 nmol/g. These results taken together suggest that the volume of distribution of cyclic AMP was reduced significantly in shock, but the rate of clearance was not.

Arterial metabolite levels

Plasma glucose (Fig. 5b) showed principally the same pattern as the cyclic AMP. Thus, in arterial rats, the levels increased during the early part of the hypotensive period, but were reduced below control values in later stages of hypotension. 6-OH-DA treatment tended to reduce the plasma glucose levels. On the other hand, demedullation alone or in combination with sympathectomy prevented the rise in glucose. The secondary fall in glucose levels was not prevented, however.

The five- to six-fold rise in blood lactate levels (Fig. 5c) in the control rats subjected to bleeding was similarly unaffected by chemical sympathectomy but was blunted by demedullation with or without 6-OH-DA. However even in the rats subjected to combined demedullation and sympathectomy the rise in lactate was four-fold, above pre-bleeding values.

The arterial pyruvate levels also rose in bled rats, albeit less than the lactate concentrations (Fig. 5d). In the control rats a 3–4-fold increase was seen. This rise was decreased both

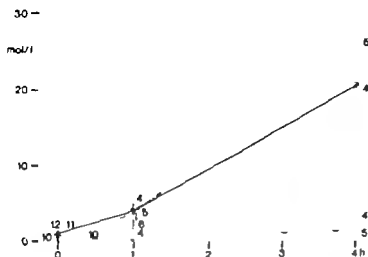


Fig. 3. Effect of bleeding on plasma noradrenaline. ●—● Controls. ○—○ Demodulated. —× Sympathectomized. □—□ Demodulated and sympathectomized. Mean \pm SE. Number of observations are given.

still above control after 4 h of hypotension (Fig. 5 a). However, in the demodulated animal cyclic AMP was almost undetectable.

In order to determine to what extent the increase in cyclic AMP may be due to decreased plasma volume and to altered elimination, the fate of i.v. administered H-cyclic AMP was determined. Following injection of H-cyclic AMP (20 nmol) the plasma level of H-radioactivity was approximately 4 times higher in bled animals than in control animals given the same amount (Fig. 6). However, the rate of disappearance of cyclic AMP was similar in shocked and control rats, since the ratio between H-cyclic AMP in the two groups of animals remained at about 4 over the entire 10-min period sampled. On the other hand, the disappearance of total radioactivity from plasma was reduced in shock. The rate of disappearance of nucleotides other than cyclic AMP and of nucleosides and bases was faster in the control animals than in animals subjected to hemorrhage (Fig. 6). Therefore, the reduced rate of H-disappearance following H-cyclic AMP injection in bled rats does not seem to be due to reduced cyclic AMP hydrolysis, but rather to reduced disappearance of the breakdown products. These findings are therefore in agreement with the presence of high activity

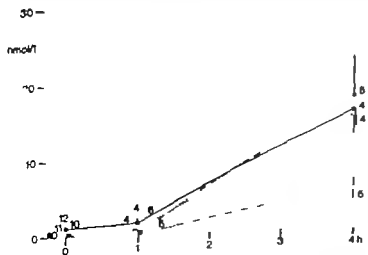


Fig. 4. Effect of bleeding on plasma dopamine. ●—● Controls. ○—○ Demodulated. —× Sympathectomized. □—□ Demodulated and sympathectomized. Mean \pm SE. Number of observations are given.

Table 2. Catecholamine levels in heart subjected to hemorrhage. The animals were untreated (control), demedullated (dem), chemically sympathectomized (6-OH DA) or demedullated and sympathectomized (dem + 6-OH-DA). A untitled control was also included. Mean \pm SE. Number of experiments in brackets. The values are expressed as $\mu\text{mol/kg}$.

Time	Animal	Control		Dem	6-OH DA	Dem + 6-OH DA
		Not bled	Bled			
1 hr	Norepinephrine		6.61 ± 0.30 (4)	7.08 ± 0.30 (6)	0.55 ± 0.05 (5)	0.49 ± 0.01 (4)
	Adrenaline		0.85 ± 0.17 (4)	ND (4)	0.09 ± 0.01 (5)	ND (4)
	Dopamine		0.05 ± 0.01 (4)	0.07 ± 0.01 (4)	0.06 ± 0.01 (5)	0.02 ± 0.01 (4)
1 hr	Norepinephrine		6.51 ± 0.31 (4)	4.87 ± 0.84 (4)	0.52 ± 0.04 (5)	0.53 ± 0.03 (5)
	Adrenaline		0.20 ± 0.01 (4)	ND (4)	0.23 ± 0.01 (5)	ND (5)
	Dopamine		0.32 ± 0.15 (4)	0.32 ± 0.06 (4)	ND (5)	0.03 ± 0.01 (5)

ND = not detectable

of cyclic nucleotide phosphodiesterase in plasma of rats (*cf* Broadbent 1977). The content of radioactivity in kidney and liver of the shock animals was lower than that of the intact animals. Thus, in kidney there was radioactivity corresponding to 1.73 ± 0.08 nmol/g in the control group and 1.27 ± 0.08 nmol/g in the shock group ($n = 3$). In the liver the corresponding figures were 0.39 ± 0.05 and 0.23 ± 0.02 nmol/g. These results taken together suggest that the volume of distribution of cyclic AMP was reduced significantly in shock, but the rate of degradation was not.

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Plasma glucose (Fig. 5b) showed principally the same pattern as the cyclic AMP. Thus, in control rats, the levels increased during the early part of the hypotensive period, but were reduced below control values in later stages of hypotension. 6-OH DA treatment tended to further reduce the plasma glucose levels. On the other hand, demedullation alone in combination with sympathectomy prevented the rise in glucose. The secondary fall in glucose levels was not prevented, however.

The five- to six-fold rise in blood lactate levels (Fig. 5c) in the control rats subjected to bleeding was similarly unaffected by chemical sympathectomy but was blunted by demedullation with or without 6-OH DA. However, even in the rats subjected to combined demedullation and sympathectomy the rise in lactate was 4-fold, above pre-bleeding values.

The arterial pyruvate levels also rose in bled rats, albeit less than the lactate concentrations (Fig. 5d). In the control rats a 3-4-fold increase was seen. This rise was decreased both

300 — cAMP

nmol

100

100

10

nmol

0

12 — Glucose

mmol

8

0

— Pyruvate

mmol

0

a

b

c

d

Fig 5 Effect of bleeding on a) plasma cyclic AMP b) plasma glucose, c) blood lactate and d) blood pyruvate. ●—● Controls. ○—○ Demedullated. Sympathectomized. □—□ Demedullated and Sympathectomized. Mean \pm SE 4-35 observations.

by sympathectomy and by demedullation and was almost prevented by the combined treatment.

The lactate/pyruvate ratio may be taken as a rough indicator of the oxygen debt (Huchbee 1958). At 4 h after hemorrhage it was 30 in the control rats, 24 in the demedullated rats and 40 in the 6-OH DA treated rats and 20 in the rats given the combined treatment. In the unbled control animals the corresponding values were 9 \pm 17 and 7 respectively. Thus in all groups of animals there was an increase in lactate/pyruvate ratio but it was less pronounced in the demedullated rats.

Finally the lipid metabolites, glycerol and FFA showed the pattern of response reported earlier for the anesthetized rat (Farnebo *et al* 1977). Thus, the glycerol levels were essentially unchanged, or even in the case of the 6-OH DA treated group decreased 1 h after bleeding (not shown). Four hours after bleeding there was a slight increase over basal in the control group (to 0.38 ± 0.06 nmol/l), but not in either of the groups of treated rats. The FFA levels were reduced to approximately half following 1 h of hypotension and remained low

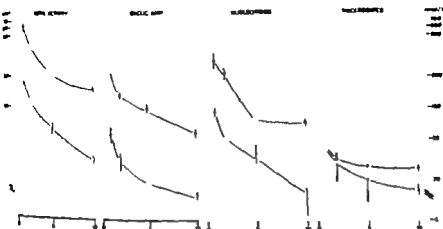


Fig. 4. Fate of ^3H -cyclic AMP in control (●—●) bled (○—○) Mean \pm SE ($n=3$). For experimental details see Methods section.

Discussion

We have used determinations of plasma catecholamines to estimate the rates of secretion. The methodological analysis presented elsewhere (Hallman *et al.* 1978), shows that the present method is applicable for determination of catecholamines in rat plasma. A problem is that the plasma catecholamine levels may be affected by alterations in the rate of disappearance or in the size of the miscible central pool. However results obtained in the dog (Watts 1965) and rat (Farnebo *et al.* 1978) show that the rate of disappearance of plasma catecholamines is similar in bled and non-bled animals. All 3 catecholamines, A, NA and DA, are removed by similar mechanisms. Therefore our finding that A levels may increase 100-fold at a time when DA levels are unchanged is further evidence that the increases in plasma catecholamine levels in shock may be largely due to increased rates of secretion. Alterations in the access to the central blood volume from which samples were taken could be a source of some error. The adrenal medulla probably has ready access to the central blood compartment. At the same time inactivation of A by uptake into sympathetic nerves may be impaired due to decreased peripheral circulation. Furthermore, the majority of NA secreting sympathetic nerves are less close to the central compartment and a greater proportion of their released A may be taken back into the nerves during bleeding than before. Therefore, the possibility exists that one may overestimate the role of the adrenal medulla if determination of relative A and NA levels is the sole criterion. However we feel that qualitatively if not quantitatively measurements of plasma catecholamines with the present technique do reflect their rates of secretion.

Bleeding induced a very marked (almost 100-fold) increase in plasma A levels after 1 h (Fig. 4). The increase in NA levels was moderate (3–4 times) during the first hour but large (10-fold) after 4 h of hypotension. The DA levels were unchanged during the first hour of hypotension but reached levels similar to those of NA after 4 h.

Denervation completely eliminated the rise in A levels following bleeding, which shows that there is practically no contribution of this amine from extra-adrenal sources. The

source of the plasma NA during hypotension is more difficult to define. After demedullation NA levels rose by 3-4 times 1 and 4 h after bleeding. This rise was almost completely prevented by chemical sympathectomy. Thus, sympathetic nerves appear to make a contribution of this magnitude to the increase in plasma NA. On the other hand, animals with chemical sympathectomy showed as large an increase in plasma NA as the intact controls after bleeding. Since a shift from A to NA secretion following prolonged stimulation of adrenal medulla has been described (Kajihara *et al.* 1977) this could mean that the large late increase in plasma NA is exclusively of adrenal origin. However, the contribution of the adrenal medulla to the plasma NA level may be over-estimated in the animals which lack the neuronal uptake mechanism for NA inactivation. Therefore it is probably safe to conclude that the plasma NA levels in late shock derive from both nerves and adrenal medulla.

During the later phase of shock there was also a very large increase in plasma DA. The origin of this DA is unclear. It was not reduced by either sympathectomy or by adrenalectomy alone. Combined sympathectomy and demedullation reduced the increase in DA but did not eliminate it. It is therefore likely that adrenal medulla, sympathetic nerves and possibly other sources all contribute. Possibly prolonged activation of sympathetic nerves and adrenal medulla activates the tyrosine hydroxylase to such a high level of activity that the dopamine β -hydroxylase reaction becomes rate limiting.

Bleeding produced extensive changes in the level plasma cyclic AMP and of circulating metabolic substrates in the anesthetized rat as described earlier (Farnebo *et al.* 1977). In order to get some idea of the relative importance of circulating amines from the adrenal medulla and noradrenaline released locally from sympathetic nerve endings we have compared the responses to bleeding in untreated, demedullated and chemically sympathectomized rats. It must be emphasized that the results were obtained in anesthetized animals and the conclusions reached here apply to this condition. Indeed, we have found that there are marked differences in the responses in anesthetized and non-anesthetized rats (Farnebo *et al.* 1978).

The rise in cyclic AMP levels following bleeding was markedly reduced by adrenal demedullation, suggesting that endogenous A is more effective than endogenous NA in raising cyclic AMP as has been shown for exogenous amines (Broadus 1977). Even in the demedullated rats and in the rats with combined sympathectomy and demedullation cyclic AMP was elevated 3-4 times 1 h after bleeding. This could mean that some stimulating factor other than catecholamines, such as glucagon (Broadus *et al.* 1970, Jerums *et al.* 1977), is responsible for this rise. Although glucagon levels are elevated in shock (Halmagri *et al.* 1969) there is increasing evidence that physiological concentrations of glucagon are sufficient to increase plasma cyclic AMP levels (*cf.* Broadus 1977). On the other hand, data with H-cyclic AMP suggest that the 4-fold rise in plasma cyclic AMP in the demedullated rats may simply be due to a decreased distribution volume.

Conversely the data strongly suggest that adrenal A is responsible for the initial rise in plasma cyclic AMP in intact and sympathectomized rats subjected to bleeding. It is therefore interesting to note that in spite of continued presence of A (and NA and DA) after 1 h of hypotension there was a progressive fall in the cyclic AMP content in plasma. An unspeci-

The depletion of the enzyme or a gradual decline in the content of ATP the substrate for cyclic AMP production, are possible explanations. A fall in tissue ATP contents during shock has been reported in several tissues of many species (Blackwood *et al* 1973, Chaudry *et al* 1973, McArdle *et al* 1975, Fredholm and Fronck 1974). Moreover Chaudry and co-workers (1977) recently reported that systemic treatment with ATP $MgCl_2$, which restores tissue ATP levels, also enhanced tissue cyclic AMP levels.

There are certain similarities between the response of plasma glucose and of cyclic AMP to hemorrhage. Thus, in the intact animals there is a rapid increase, followed by a progressive fall, down below control values. The initial increase was enhanced by sympathectomy and reduced or eliminated by demedullation. These findings suggest that in contrast to the situation in cats (Lefebvre 1975) sympathetic nerves do not play an important role in the mobilization of glucose in rats subjected to hemorrhage.

Blood levels of lactate and pyruvate also rise during hemorrhage. The time course of the rise is different from that observed for glucose, which suggests that it is not only caused by increased glucose levels. The increase in lactate was considerably attenuated by demedullation, but was essentially unaffected by 6-OH DA treatment. The rise in pyruvate was diminished by demedullation as well as by chemical sympathectomy and particularly by the adrenalectomy. This suggests that at least part of the rise in lactate and pyruvate is due to release of catecholamines. However also in the rats with combined demedullation and adrenal sympathectomy there was a rise in lactate and pyruvate, and in the lactate/pyruvate ratio. Although the influence of endogenous catecholamines is not completely eliminated by the combined treatment the data suggest that the hypotensive shock *per se* alters the lactate and pyruvate metabolism. Studies of anaphylactic shock in rabbits also suggested that lactate and pyruvate levels might change without the mediation of catecholamines (Fredholm and Strandberg 1975). It has been suggested that lactate and pyruvate levels in plasma may be indicators of the severity of shock (Well and Affl 1970). The present results indicate that the relationship may be less than perfect. For example, adrenal demedullation, which is known to decrease survival (Chien 1967) decreased rather than increased lactate and pyruvate levels.

During the later phases of hypotension the animals took back fluid from the reservoir. This is usually ascribed to decompensation, leading into an irreversible phase of shock. The reasons for this irreversibility has been much speculated over and one possibility is that it is due to a failure of the sympatho-adrenal system (*cf* Chien 1967). The present data clearly indicate that decompensation may occur even if the secretion of catecholamines is kept high. Similarly Farnebo and Hamberger (1977) found little evidence that sympathetic nerves in shock have a deficient capacity to take up or release NA in shock. Thus a failure of the sympatho-adrenal system *per se* is an unlikely explanation for irreversibility at least in anesthetized rats.

It is interesting that the decompensation phase following bleeding is characterized by low cyclic AMP and glucose levels in plasma. Furthermore, during this late phase of hypotension the FFA levels are depressed. Thus the level of metabolic substrates is low and there is evidence that the mechanisms supporting increased metabolism (e.g. cyclic AMP) are also depressed. Therefore the failure of the homeostatic mechanisms during shock may

be due to failing metabolism rather than failing sympatho-adrenal activity. Ultimately metabolic failure might (via e.g. ATP-depletion and acidosis) lead to a failure in its responsiveness to catecholamines.

The rate at which fluid was taken back during the decompensation phase was reduced by sympathectomy irrespective of whether the adrenal medulla was present or not. This finding could suggest that in fact the sympathetic nervous system contributes to the irreversible alterations leading to irreversible shock. This is interesting in view of the considerable experimental evidence that pretreatment with α -adrenergic blocking agents may be beneficial in preventing irreversible shock (see Chien 1967). There are at least two explanations for the apparently beneficial effect of combined demedullation and sympathectomy and of pretreatment with α -adrenoceptor antagonists. Firstly the blood loss is reduced. Secondly the degree of peripheral vasoconstriction may be reduced with consequent better tissue perfusion. If the differences in bleed volume at 1 hr are taken into account, the most prominent feature during the late phase of bleeding is the remarkable decompensation in the demedullated animals. This is probably due to the combined action of long standing vasoconstriction due to the active sympathetic nervous influence and lack of metabolic substrate leading to severely compromised tissue function.

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References

- BALLO P. del, G. RUCCA and A. CARMI, Drug-induced changes of urinary catecholamines in the rat: Role of the adrenal medulla. *Eur. J. Pharmacol.* 1970. 13 83-89.
- BLACKWOOD, J. M. J. HUGHES, J. FEWELL and B. F. RUSSELL, Jr. Tissue metabolites in endotoxaemia and hemorrhagic shock. *Arch. Surg.* 1973. 107 181-185.
- BROADBENT, A. E., Clinical cyclic nucleotide research. *Adv. Cyclic Nucleot. Res.* 1977. 8 509-544.
- BROADBENT, A. E., N. I. HAMSHAW, R. C. NORTHCUTT, J. G. HARDMAN, E. W. SUTHERLAND and G. LIDDLE, Effects of glucagon on adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in human plasma and urine. *J. clin. Invest.* 1970. 49 237-245.
- BROWN, B. L., R. P. EKINS and J. D. ALBANO, Saturable assay for cyclic AMP using endogenous bound protein. *Adrenal Cyclic Nucleot. Res.* 1972. 2 25-41.
- CHAM, P. S. and M. C. LEE, Isolation of cyclic AMP by inorganic salt coprecipitation. In *Methods in enzymology* XXXVIII (Eds. J. G. Hardman and B. W. O. Malley). New York 1974. Pp. 38-41.
- CHAUDRY, I. H., M. M. SAYEED and A. E. BAUS, Effect of hemorrhagic shock on tissue adenosine nucleotides in conscious rats. *Can. J. Physiol. Pharmacol.* 1974. 56, 131-137.
- CHAUDRY, I. H., K. A. WICHTERMAN and A. E. BAUS, Restoration of cellular adenosine 3',5'-monophosphate levels by ATP $MgCl_2$ in hemorrhagic shock. *Fed. Proc.* 1977. Abstr. 715.
- CHEN, S., Role of the sympathetic nervous system in hemorrhage. *Physiol. Rev.* 1967. 47 214-242.
- ENGEL, F. L., M. G. WINTON and C. N. H. LOWE, Biochemical studies on shock. I. The metabolism of amino acids and carbohydrate during hemorrhagic shock in the rat. *J. exp. Med.* 1943. 77 397-410.
- FARNEBO, L.-O., H. HALLMAN, B. HAMBERGER and G. JOHANSSON, Catecholamines and hemorrhagic shock in awake and anesthetized rats. Submitted to *Circulatory Shock*.
- FARNEBO, L.-O. and B. HAMBERGER, Noradrenergic mechanisms in hemorrhagic shock of the rat. *Acta physiol. scand.* 1977. 143 1-8.
- FARNEBO, L.-O., B. B. FREDHOLM, B. HAMBERGER, B. HJERNDAL and L. WESTMAN, Cyclic AMP and metabolic substrates in hemorrhagic shock of the rat. *Acta chir. scand.* 1977. 143 9-14.
- FREDHOLM, B. B. and A. FRONK, High energy phosphat compounds in adipose tissue. The effect of hemorrhage. *Acta physiol. scand.* 1974. 91 165-171.

- FRANKLIN, B. B. and K. STEINBERG, Some metabolic consequences of the anaphylactic reaction in the rabbit. *Acta physiol. scand.* 1975. 93: 52-58.
- KURT, E. W. Survival of adrenalectomized Long-Evans and Sprague Dawley rats. *Laboratory Animal Care* 1969. 19: 774-726.
- KULLMAN, H., L.-O. FARNEBO, B. HANSSON and G. JOHANSSON, A sensitive method for the determination of plasma catecholamines using liquid chromatography with electrochemical detection. *Life Sci.* 1978. 23: 3049-3052.
- KULMAVI, D. F., J. M. H. BROWN and D. J. OLLETT, Course of posthemorrhagic hypotension after aortic ganglionectomy with and without adrenalectomy. *Ann. Surg.* 1967. 166: 222-227.
- KULMAVI, D. F., J. L. R. NIERHO, L. LAZARUS, J. D. YOUNG and J. PULLIN, Plasma glucagon in experimental posthemorrhagic shock. *J. Trauma* 1969. 9: 320-326.
- MCNETT, J. M., Z. CILIX, J. S. SOULENER and R. M. ENODANT, Insulin response to hemorrhagic shock in the intact and adrenalectomized primate. *Amer. J. Surg.* 1973. 125: 301-307.
- RELMER, W. E., Relationship of pyruvate and lactate during anaerobic metabolism. I. Effect of infusion of pyruvate, of glucose and hyperventilation. *J. clin. Invest.* 1958. 37: 244-254.
- RESCIT, J. Osmotic control of the circulation in hemorrhagic hypotension. *Acta physiol. scand.* 1975. Suppl. 423: 1-84.
- SAMRA, H., S. HIRA and N. MIYOSHI, Changes in blood catecholamine levels and ultrastructure of dog adrenal medullary cells during hemorrhagic shock. *Verh. Arc. B. Cell Path.* 1977. 21: 1-16.
- SEILER, R., A. OEL, I. MUECKE and R. N. ADAMS, Liquid chromatographic analyses of catecholamines: simple assay for regional brain mapping. *Life Sci.* 1976. 19: 995-1004.
- LACCELL, S. and G. TOSOLINO, An enzymatic fluorimetric micromethod for the determination of glycerol. *Chim. Acta* 1966. 12: 517-523.
- LACCELL, S. and G. TOSOLINO, Colorimetric micromethod for free fatty acids in plasma. *Chim. Acta* 1967. 14: 57-6.
- LIVEMORE, S. M., A. ENGLISH and M. J. MALM, Nutritional and metabolic aspects of shock. *Fed. Proc.* 1964. 23 Suppl. 9: 99-140.
- MCALPIN, A. H., C. J. CHU and J. HICKS, Cyclic AMP response to epinephrine in shock. *Arch. Surg.* 1975. 106: 516-520.
- MORROW, L., A. OEL, R. N. ADAMS and G. JOHANSSON, Epinephrine localization in brown adipose tissue. *Neuroscience Letters* 1977. 5: 141-145.
- WATTS, D. T. Adrenergic mechanisms in hypovolemic shock. In: *Shock and Hypotension* (Eds. L. C. Mills and J. K. Meyer). New York 1965. 385-391.
- WIS, M. H. and A. A. ARPS, Experimental and clinical studies on lactate and pyruvate as indicators of the severity of acute circulatory failure (shock). *Circulation* 1970. 41: 949-1001.
- WILLIAMS, H. and M. H. WEX, A model for the study of hemorrhagic shock in the rat. Development of the method. *J. appl. Physiol.* 1966. 21: 1840-1863.

be due to failing metabolism, rather than failing sympatho-adrenal activity. Ultimately (a metabolic failure might (via e.g. ATP-depletion and acidosis) lead to a failure in tissue responsiveness to catecholamines.

The rate at which fluid was taken back during the decompensation phase was reduced by sympathectomy irrespective of whether the adrenal medulla was present or not. This finding could suggest that in fact the sympathetic nervous system contributes to the irreversible alterations leading to irreversible shock. This is interesting in view of the considerable experimental evidence that pretreatment with α -adrenergic blocking agents may be beneficial in preventing irreversible shock (see Chien 1967). There are at least two explanations for the apparently beneficial effect of combined demedullation and sympathectomy and of pretreatment with α -adrenoceptor antagonists. Firstly the blood loss is reduced. Secondly the degree of peripheral vasoconstriction may be reduced with consequent better tissue perfusion. If the differences in bled volume at 1 hr are taken into account, the most prominent feature during the late phase of bleeding is the remarkable decompensation in the demedullated animals. This is probably due to the combined action of long standing vasoconstriction due to the active sympathetic nervous influence and lack of metabolizable substrate leading to severely compromised tissue function.

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References

- BASSO, P. del, G. RUSCA and A. CARPI, Drug-induced changes of urinary catecholamines in the rat: Role of the adrenal medulla. *Europ. J. Pharmacol.* 1970, 13, 83-89.
- BLACKWOOD, J. M., J. HATCH, J. FEWEL and B. F. RUSH, Jr. Tissue metabolism in endotoxic and hemorrhagic shock. *Arch. Surg.* 1973, 107, 181-185.
- BROADUS, A. E., Clinical cyclic nucleotide research. *Adenine Cyclic Nucleotides* 1977, 8, 509-548.
- BROADUS, A. E., N. I. KAMINNEY, R. C. NORTHCUTT, J. G. HARDMAN, E. W. SUTHERLAND and G. W. LINDLE, Effects of glucagon on adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate in human plasma and urine. *J. In. Invest.* 1970, 49, 237-245.
- BROWN, B. L., R. P. EKER and J. D. ALBANO, Saturation assay for cyclic AMP using endogenous binding proteins. *Adenine Cyclic Nucleotides* 1972, 2, 25-41.
- CHAM, P. S. and M. C. LYN, Isolation of cyclic AMP by inorganic salt coprecipitation. In *Methods in Enzymology* XXXVIII (Eds. J. G. Hardman and B. W. O. Malley). New York 1974, Pp. 38-41.
- CHAUDRY, I. H., M. M. SAYED and A. E. BAUR, Effect of hemorrhagic shock on tissue adenosine nucleotide in conscious rats. *Can. J. Physiol. Pharmacol.* 1974, 56, 131-137.
- CHAUDRY, I. H., K. A. WICHTERMAN and A. E. BAUR, Restoration of cellular adenosine 3',5'-monophosphate levels by ATP $MgCl_2$ in hemorrhagic shock. *Fed. Proc.* 1977, Abstr. 715.
- CHEN, S., Role of the sympathetic nervous system in hemorrhage. *Physiol. Rev.* 1967, 47, 214-233.
- ENGL, F. L., M. K. WINTON and C. N. H. LOVE, Biochemical studies on shock. I. The metabolism of amino acids and carbohydrate during hemorrhagic shock in the rat. *J. exp. Med.* 1943, 77, 397-410.
- FARNEBO, L.-O., H. HALLMAN, B. HAMBERGER and G. JONSSON, Catecholamines and hemorrhagic shock in awake and anesthetized rats. Submitted to *Circulatory Shock*.
- FARNEBO, L.-O. and B. HAMBERGER, Noradrenergic mechanisms in hemorrhagic shock of the rat. *Acta chir. scand.* 1977, 143, 1-8.
- FARNEBO, L.-O., B. B. FREDHOLM, B. HAMBERGER, P. HEMDAHL and L. WESTMAN, Cyclic AMP and metabolic substrates in hemorrhagic shock of the rat. *Acta chir. scand.* 1977, 143, 9-14.
- FREDHOLM, B. B. and A. FROBERG, High energy phosphate compounds in adipose tissue: The effect of hemorrhage. *Acta physiol. scand.* 1974, 91, 163-171.

of the content of a vesicle. Nevertheless, if the vesicles were in open communication with the synaptic cleft during the transmission process, both total and partial emptying of a vesicle could result in a parallel release of all components stored in it, such as NA, ATP, proteins and sulphomucopolysaccharides (SMPSs).

Sjöström *et al.* (1970) reported that stimulation of the splenic nerve of the cat resulted in a release of NA but not of ATP. Such a selective release of NA cannot be explained by a simple theory of complete exocytosis. *In vitro* studies in our laboratory have shown that vesicle material from adrenomedullary cells (Uvnäs and Åborg 1977) and splenic nerves (Uvnäs *et al.*, to be published) possesses properties of a cation exchange material capable of binding catecholamines even in the absence of ATP. These findings suggest the possibility that a selective fractionate release of the noradrenergic transmitter might be due to a nerve impulse-induced cation exchange over the depolarized nerve terminal membrane (Uvnäs 1979). As one step on our way to clarifying the question of a selective transmitter release we decided to study quantitative changes of the noradrenergic vesicle components NA, ATP and SMPSs in subcellular fractions from the cat spleen during splenic nerve stimulation.

We found that nerve stimulation in the presence of phenoxybenzamine and imipramine induced a significant fall of NA contents in the noradrenergic vesicle fractions but did not lower the contents of ATP and ^{35}S -labelled SMPSs.

Methods

Experimental. Cats of both sexes (2.5–4 kg) were anaesthetized subcutaneously with $\text{Na}_2\text{S}_2\text{O}_5$ (carrier-free, Lach, Norway) in a dose of 8 mg/kg. They were anaesthetized 24 h later with sodium pentobarbital (60 mg/kg) or chloralose (90 mg/kg). The body temperature was kept 36–37°C by means of heating pad. Heparin (400 IU/kg) was given. Blood pressure was recorded from the common carotid artery via Statham transducer on Grass polygraph. The abdomen was opened by midline incision. The vessels in the meso (frontal) portion of the spleen were ligated and the spleen was clamped tightly at the incision between the meso and wide (dorsal) portion. The frontal portion—serving as control—was first removed, cut into small pieces, rinsed with ice-cold sucrose, blotted dry and put on ice. Phenoxybenzamine (PBA), Dulcoryline, Scatch, Klose and French), 10 mg/kg, and imipramine (Tofranil, Geigy), 1 mg/kg, were injected during 20 min each. The splenic nerve was prepared for stimulation. Electrical stimulation of the splenic nerve was carried out with shielded platinum electrodes connected to 4D Grass stimulator, at supramaximal pulses of 2 ms duration at frequency of 10 Hz. During 30 min total of 1800 pulses was applied. Immediately thereafter the remaining portion of the spleen was removed and rinsed the same way as the control. The clamped piece was discarded.

Subcellular fractionation. Both portions of the spleen were subjected to the same fractionation procedure. They were chopped and homogenized in 5 volumes of ice-cold 0.05 M sucrose, containing 0.001 M MgCl_2 and 0.05 M potassium phosphate buffer pH 7.4, in an Ultra Turrax apparatus at 100 V for 1 min. The homogenates were centrifuged at 15 000 g_{max} for 15 min and the resulting supernatants were layered onto discontinuous gradients of 16%, 12%, 8%, 4% and 2% Ficoll (Pharmacia, Uppsala) in 0.25 M sucrose. By bottom pellet and 5 bands, visible at the borderlines between the different Ficoll solutions, are obtained together with the adjacent Ficoll, diluted with isotonic sucrose and centrifuged at 125 000 g_{max} for 30 min. The final pellets were resuspended by hand in 0.25 M sucrose. The particulate fractions are listed in gradient fraction 1–6 (starting at the top of the gradient). Aliquots of the fractions were taken for determination of NA, ATP, protein, total and lipid-bound ^{35}S , in a few experiments also for cytochrome-C-oxidase (Cy-C-Ox) and glucose-6-phosphatase (G-6-pase).

NA. NA was extracted from tissue fractions with 0.4 N HClO_4 , separated by chromatography on Al_2O_3 and assayed by the trihydroxyindole method (Udenfriend 1962). The recovery of NA was 95–103%. Protein was determined according to Lowry *et al.* (1951). ATP was extracted from the fractions by boiling in 10 M sucrose for 10 min in a water bath. The precipitated protein was spun down and the ATP deter-

Effect of splenic nerve stimulation on the contents of noradrenaline, ATP and sulphomucopolysaccharides in noradrenergic vesicle fractions from the cat spleen

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Abstract

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In order to assess the effect of splenic nerve stimulation on the contents of noradrenaline, ATP and 35 S-labelled compounds in noradrenergic vesicles from the spleen, experiments were carried out on the cat spleen *in situ*. Its frontal portion being used as control. Particulate fractions containing noradrenergic vesicles were isolated on Ficoll gradient centrifugation of homogenates from both portions of the spleen. The cats received phenoxybenzamine (10 mg/kg) and mipramine (5 mg/kg) to inhibit the contractile response of the spleen to nerve stimulation and the reuptake of noradrenaline (NA) into nerve terminals. Administration of the drugs did not significantly affect the levels of NA, ATP and 35 S (presumably in sulphomucopolysaccharides, SMPs). In response to prolonged stimulation of the splenic nerve a significant loss of NA from noradrenergic vesicles occurred, whereas levels of ATP and 35 S-labelled compound remained unchanged. The results indicate that a discharge of NA in response to nerve stimulation is not accompanied by simultaneous discharge of ATP and SMPs.

According to current opinion, transmitter release at nerve terminals occurs as a nerve impulse-induced quantal secretion. It has been proposed (for references see Hubbard 1970) that the released quantal package corresponds to the content of a transmitter storage vesicle. However, comparative calculations of the transmitter content in varicosity vesicles and the amount of transmitter released per nerve impulse have cast doubts on the validity of a simple vesicle theory, especially as regards noradrenergic transmission. For example, nerve impulse-induced output of noradrenaline (NA) from a varicosity in the rat iris has been estimated to correspond to only a small fraction (400 molecules of NA) of the content of one vesicle (15 000 molecules of NA) out of 1 000 vesicles in the varicosity (Folkow *et al.* 1967). If these calculations are correct, the transmitter quantum might be only a fraction

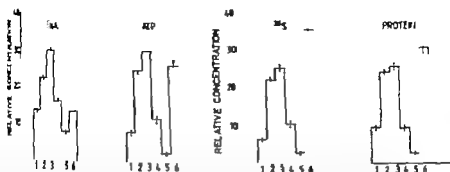


Fig. 1 Distribution of NA, ATP, ^{55}S and protein on Ficoll gradients. Particulate fractions of splenic homogenates were isolated on Ficoll gradient centrifugation from control portions of the spleen ($n=14$), described in Methods. NA, ATP, ^{55}S and protein in fractions 1-6 are expressed as percentages of their content in the gradient. Means \pm S.E. are given.

Results

Distribution of NA, ATP, ^{55}S , protein and marker enzymes on Ficoll gradients from control portions of the spleen

The particulate gradient fractions 1-6 were examined. Levels of endogenous NA, which presumably reflect the distribution of noradrenergic vesicles, were highest in fractions 2 and 3 (50% of the total), while fractions 1 and 4 contained only about 15% each (Fig. 1 a). The distribution of ATP, total ^{55}S and protein were similar to the NA pattern (Fig. 1 b-d). The bottom pellet (fraction 6) with high contents of ATP, ^{55}S and protein, was macroscopically heterogeneous due to heavy conglomerates of particles. This fraction also showed the highest contamination with Cy-C-Ox (Fig. 2 a), while G-6-pase was distributed rather evenly among all gradient fractions (Fig. 2 b). Electron microscopic examination of the fractions (unpublished results) and the distribution of the enzymic markers supported the presumption that the major part of noradrenergic vesicles occurred in fractions 1-4 and was contaminated heavily with microsomes but only slightly with mitochondria.

Lipid-bound ^{55}S represented 20% (fractions 1, 2 and 4) and 10% (fraction 3) of the total ^{55}S radioactivity in the corresponding fraction (Table II). In other words, 80-90% of the ^{55}S was incorporated into non-lipid compounds. Therefore, the distribution of total ^{55}S

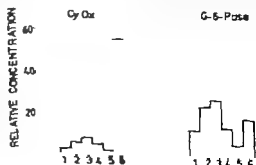


Fig. 2 Distribution of Cy-C-Ox and G-6-pase on Ficoll gradients. Experimental procedures and presentation of results as in Fig. 1. Total enzymic activities on the gradient were: for Cy-C-Ox 1.35 Δ log ferrocyanochrome (cm) for G-6-pase 3.9 nmol isoenzyme (phosphate min).

mined in the supernatant fluid by the luciferine-luciferase method essentially according to Stanley and Williams (1969) with modifications previously described (Fredholm *et al.* 1977). The extraction (checked by reextractions) was nearly complete. The recovery of ATP in the assay was $94.7 \pm 2.6\%$. The total radioactivity was determined in aliquots of the resuspended fractions after addition of 10 ml laseq (Packard) by liquid scintillation spectrometry in a Packard Tricarb counter⁹. Correction for quench was made using an external standard system. Lipid-bound ³⁵S was extracted from the fractions overnight over phosphorus pentoxide by repeated homogenization and centrifugation in chloroform-methanol (2:1 v/v). After evaporation of the solvent, 10 ml of a toluene scintillation liquid were added and the radioactivity due to ³⁵S was determined. Non-lipid bound ³⁵S was expressed as the difference between total and lipid-bound ³⁵S.

Cy-C-Ox activity was determined according to Cooperstein and Lazaro (1951). G-6-pase activity assayed by the slightly modified method of Svanesson (1955), the inorganic phosphate according to Fiebigg and Gubareff (1965).

Statistics. Results are expressed as means \pm S.E. Hypotheses were tested by Student's *t*-test.

CONCLUSION

The frontal portion of the spleen from the same animal was used as control in order to avoid striations in NA contents of spleens from different cats (Dearnaley and Geffen 1966). While some authors found differences in sympathetic innervation (Green and Fleming 1968) or NA contents (Dearnaley and Geffen 1966) between the thinner and thicker portions, others did not (Hedqvist and Stjärne 1969). In accordance with the latter authors we found equal amounts of total particle-bound NA in the two portions of the organ (Fig. 4). Therefore it seems highly preferable to evaluate effects of pharmacological manipulation and electrical nerve stimulation by comparing portions of the same spleen, instead of comparing groups of control and treated animals, as done by Chubb *et al.* (1972) and by Flienz and Howe (1971).

The homogenates were centrifuged at 15 000 *g*_{max} for 15 min, in order to diminish contamination of noradrenergic vesicles by mitochondria. It has been argued (De Potter *et al.* 1977; with Bosby and Fiebigg 1971) that such a centrifugation causes a significant loss of one population of noradrenergic vesicles, the dense ones. On checking the yield of noradrenergic vesicles from the rat heart and spleen after centrifugation in the range 6 000–15 000 *g*_{max} (unpublished results) we found no significant differences in the yields. Neither of course did this centrifugation remove all mitochondria.

We achieved an 18-fold purification of noradrenergic vesicles from the homogenate, with values of about 150 ngNA/mg protein. This is not very high compared to maximal values of 720 ngNA/mg protein in fractions isolated from the cat spleen by double gradient technique (Bosby and Fiebigg 1971). Since in our experiments the effects were evaluated by comparing corresponding noradrenergic vesicle fractions before and after treatment, we considered it more important to avoid a loss of vesicles than to achieve high purity. Such a loss inevitably occurs during repeated purification procedures (unpublished results).

Drugs were administered before nerve stimulation in order to prevent contraction of the spleen and release of released NA. PBA is considered to block postsynaptic α -receptors (Nickerson 1957) as well as the uptake of NA into nerve terminals (for references see Iversen 1967) and, via blockade of presynaptic α -receptors, to enhance the release of NA and dopamine- β -hydroxylase (DBH) during stimulation (for references see Stjärne 1976). Since a complete blockade of NA reuptake was desirable and a rise of the PBA dose had a strong depressant effect on the systemic blood pressure, imipramine was used to reinforce the blockade of the reuptake of NA (for references see Iversen 1967). Since the contents of the noradrenergic vesicle fractions might be influenced by the drugs, their effect was investigated separately.

The influence of drug treatment and nerve stimulation was evaluated by comparing the contents of NA, ATP and ³⁵S-labelled compounds in the particulate gradient fractions from the control portion with the corresponding values in the fractions from the treated portion of the spleen. Since the amounts of tissue in the two portions were different, absolute values could not be compared. Instead, the amount of protein in each fraction was used as reference substance. The amount of the substance (NA, ATP or ³⁵S-labelled material) per mg protein in each fraction from the dorsal portion was expressed as a percentage of the amount of the substance per mg protein in the corresponding fraction from the frontal portion. So an evaluation seemed justified, because a) no significant redistribution of protein was observed after drug treatment and nerve stimulation, b) in all experiments the recovery of particle bound protein and NA on the gradients from the respective homogenates was approximately the same for both portions (Table 1). Although a loss of particulate protein from noradrenergic vesicles during nerve stimulation cannot be ruled out, such a change appears negligible compared to the total mass of particulate protein in the fractions.

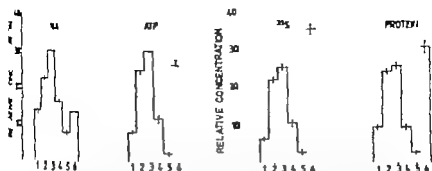


Fig. 1 Distribution of NA, ATP ³⁵S and protein on Ficoll gradients. Particulate fractions of splenic liposomes were isolated on Ficoll gradient centrifugation from control portions of the spleen ($n=14$), isolated as Methods. NA, ATP ³⁵S and protein in fractions 1-6 are expressed as percentages of their total in the gradient. Means \pm S.E. are given.

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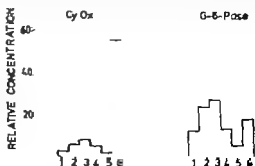


Fig. 2 Distribution of Cy-C-Ox and G-6-Pase on Ficoll gradients. Experimental procedure and presentation of results as in Fig. 1. Total enzyme activities on the gradient were: for Cy-C-Ox 1.35 Δ log ferrocyanide/min, for G-6-Pase 3.9 nmol/min/mg protein/min.

TABLE 1 Recovery of particulate protein and NA on the Ficoll gradient. Particulate fractions of the splenic homogenate were isolated as described in Methods. The sum of protein and NA in all 6 gradient fractions is expressed as a percentage of the total protein in the respective homogenates. F=frontal portion, D=dorsal portion of the spleen. Means \pm S.E. are given (n=25). N.S.=not significant.

	Protein		Noradrenaline	
	F	D	F	D
% of homogenate	2.6 \pm 0.2	2.4 \pm 0.2	14.7 \pm 1.2	N.S. 18.9 \pm 1.3

In these fractions may be considered representative for the distribution of non-lipid compounds, presumably SMPSs (and sulphated glycoproteins). It appeared to follow closely the distribution of NA (Fig. 1c and a).

Influence of drug treatment and splenic nerve stimulation

Three types of experiments were performed: 1) In control experiments both portions of the spleen were removed within an interval of about 1½ h, without any treatment of the cat. 2) In drug treatment experiments PBA and Imipramine were administered during this interval. 3) In nerve-stimulation experiments the splenic nerve was stimulated 20–30 min after the end of drug administration.

The percentual changes of NA, ATP and 35 S-labelled compounds in gradient fractions 1–4 and in the sum of fractions 1–6 were compared between control and drug-treatment, and drug treatment and nerve-stimulation experiments, respectively (Fig. 3a–d and 4). Comparison of fractions 5 and 6 was omitted because of low contents of NA and heavy contamination with various subcellular particles, respectively. NA, ATP and 35 S contents of corresponding gradient fractions (1–4) from untreated frontal and dorsal portions of the spleen (control experiments) varied between 80 and 120%. The variations were probably mainly due to methodological inaccuracies during gradient fractionation. After treatment of the animals with PBA and Imipramine, no significant changes were observed in the NA and 35 S contents of the fractions. Except for fraction 1 there was consistently a slight decrease in the ATP contents of the fractions. In the drug treated spleen, stimulation of the splenic nerve induced a significant reduction of NA to 46–50% in fractions 1–4 as well as when expressed for the sum of fractions 1–6 but no change in the contents of ATP and 35 S-labelled compounds.

TABLE II Contents of lipid-bound 35 S in Ficoll gradient fractions 1–4. Cats were injected s.c. with $\text{Na}_2^{35}\text{SO}_4$ 24 h before removal of the spleen. Particulate fractions from the splenic homogenate were isolated as described in Methods. From each fraction lipid-bound 35 S was extracted with chloroform-methanol. Lipid bound 35 S is expressed as a percentage of the total 35 S in each fraction. Means \pm S.E. are given (n=10).

	F 1	F 2	F 3	F 4
% of total 35 S	17.5 \pm 1.9	13.4 \pm 1.8	9.6 \pm 0.8	16.4 \pm 1.3

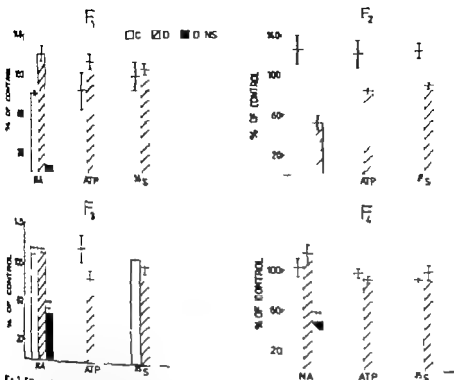


Fig. 2. Effect of drug treatment and nerve stimulation on contents of NA, ATP and 45 S in FicolI gradient fractions F₁-F₄. Particulate fractions containing noradrenergic vesicles were isolated from frontal and basal portions of the spleen in control (C), drug-treatment (D) and nerve-stimulation (D+NS) experiments, as described in Methods. PBA 10 mg/kg and amphetamine 5 mg/kg were administered i. in both D and D+NS experiments, in the latter the splenic nerve was stimulated (sh 10 Hz for 30 sec). NA, ATP and 45 S per mg protein in each fraction from the dorsal portion were expressed as percentages of the corresponding values from the control frontal portion. Means \pm S.E. are given. of C=3, of D=4, of D+NS=7. Student's *t*-test was applied for comparison between C, D and D+NS experiments. p 0.001 - 0.0025 p < 0.01.

The NA/ATP molar ratios (Table III) in gradient fractions from control and drug-treated spleen had values between 1.7 and 4.8. Stimulation of the splenic nerve in the drug-treated spleen was followed by a significant fall of the NA/ATP ratios, most pronounced in fraction 4. The close correlation of the percentual changes in total and non-lipid 45 S in the drug-treated experiments (Table IV), together with the prevalence of non-lipid 45 S over lipid 45 S in the fractions (Table II), support the assumption that changes of total 45 S in the nerve stimulation experiments reflect mainly changes of non-lipid 45 S (which for technical reasons were not measured directly). Since no changes of total 45 S were observed after nerve stimulation (Fig. 3 a-d, 4) we may assume that the contents of non-lipid 45 S were not affected, etc.

Discussion

The distribution of noradrenergic vesicles on the FicolI density gradient (used in order to avoid hypertonic media) differed from the pattern usually reported for sucrose gradients. On the latter two peaks of NA were found at 1.0 and 1.4 g/ml sucrose on isopycnic centrifuga-

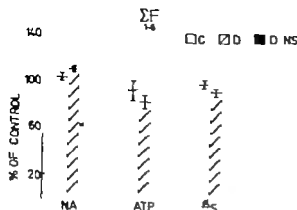


Fig. 4 Effect of drug treatment and nerve stimulation on contents of NA, ATP and ^{35}S in the sum of particulate gradient fractions 1-4. For explanation see Fig. 3

tion of microsomal pellets from the dog spleen (Chubb *et al* 1970), and at 0.7 M and 1.1 M sucrose in the cat spleen (Blaby and Fillenz 1971). In a few experiments we relayered subcellular fractions of the rat spleen from Ficoll gradients onto continuous sucrose gradients: fraction 1 sedimented mainly at 0.5 M, fractions 2 and 3 at 1.1-1.2 M and fraction 4 at 1.5 M sucrose (unpublished results). Although discontinuous gradients do not provide information as to the equilibrium density, it seems that by means of Ficoll gradients several populations of NA-containing particles may be separated, possibly on the basis of other characteristics beside density. The existence of several populations of NA-containing particles has been indicated by electron microscopic observations (Tranzer 1973), and by the presence of 3-4 peaks of particulate NA on sucrose gradients, when homogenates from the dog spleen were, after low precentrifugation, directly layered on the gradients (Nelson and Molinoff 1976). These vesicle populations were characterized by different NA/DBH ratios.

The concomitant distribution of NA and ATP in fractions 1-4 agrees well with the known presence of ATP in noradrenergic vesicles (for references see Lagercrantz 1976). The parallel distribution of NA with ^{35}S -labelled material in fractions 1-4 confirms our previous findings on various other types of gradients (Åborg *et al* 1972, Blaschke and Uvnäs 1972). Since more than 80% of the ^{35}S -labelled material was incorporated into non lipid compounds, *i.e.* SMPSs and possibly sulphated glycoproteins (Robinson

TABLE III Effect of nerve stimulation (NS) on the NA/ATP ratio in the Ficoll gradient fractions 1-4. After removal of the frontal portion of the spleen, cats received PBA 10 mg/kg and imipramine 5 mg/kg and the splenic nerve was stimulated with 10 Hz for 30 min. Then the dorsal portion of the spleen was removed. Particulate fractions of the homogenates were isolated as described in Methods. NA/ATP ratios from control and drug-treated portions of the spleen are given in row 1. NA/ATP ratios from the control portions (row 2) were compared with the corresponding ratios from the drug treated and nerve-stimulated portion (row 3) by the paired *t* test. Means \pm S.E. are given ($n=7$). $-p<0.05$ $-p<0.01$ $-p<0.005$.

	P 1	P 2	P 3	P 4
Control and drug treated spleen	4.1 \pm 0.4	4.0 \pm 0.1	2.7 \pm 0.2	3.2 \pm 0.2
Before NS (-frontal portion)	4.8 \pm 1.1	1.7 \pm 0.2	1.8 \pm 0.1	3.0 \pm 0.2
After NS (-dorsal portion)	2.3 \pm 0.6	*0.8 \pm 0.2	*0.8 \pm 0.0	1.9 \pm 0.2

TABLE IV Comparison of the effect of drug treatment on the total and non-lipid ^{45}S in the Picoil gradient fractions. Cats were anaesthetized with $\text{Na}_2\text{S}_2\text{O}_8$ and I. with PBA (10 mg/kg) and imipramine (5 mg/kg) 24 h and 2 h before removal of the spleen, respectively. Particulate fractions of the splenic homogenates were isolated, and total and lipid-bound ^{45}S determined as described in Methods. Non-lipid ^{45}S = total ^{45}S - lipid-bound ^{45}S . ^{45}S per μg protein in each fraction from the decal portion is expressed as percentage of the corresponding value from the control frontal portion. Means \pm S.E. are given. The effect on total and non-lipid ^{45}S was correlated $r=0.79$ significant at $p=0.01$.

	F 1	F 2	F 3	F 4	F 5	F 6
Total ^{45}S	104.1	88.8	98.8	98.1	76.4	80.5
% of control	± 3.4	± 3.1	± 6.7	± 7.5	± 7.3	± 5.1
Non-lipid ^{45}S	184.9	78.9	94.3	83.6	78.6	81.6
% of control	± 19.3	± 1.2	± 6.6	± 7.7	± 7.2	± 8.7

(Gross 1962, Margolis and Margolis 1970), the results further support our previous evidence that SLPs are true components of the noradrenergic vesicle (Blaschke *et al.* 1976). The distribution of ^{45}S -labelled lipids corresponded approximately to the pattern of the microsomal marker G-6-pase, in agreement with Lagercrantz (1971), who found more lipids in microsomal fractions than in noradrenergic vesicle fractions. A part of the lipid- ^{45}S might be, however, incorporated into sulphatides, which have been shown to be present in noradrenergic vesicles from bovine splenic nerves (Blaschke *et al.* 1976).

Up to 2 h after the I. administration of PBA and imipramine, we observed no significant changes of NA levels in the noradrenergic vesicle fractions. The finding is at variance with the results of Falkert and Howe (1975), who observed a 50% decrease of splenic NA levels in anaesthetized rats with PBA. However their dose of PBA was 2.5 times higher and the time which elapsed after administration of the drug was longer—moreover since the rats were intact, their sympathetic activity could have been increased reflexively following the α -receptor blockade (Dooten and Nickerson 1957). Our findings are, on the other hand, in good agreement with the observations of Hedqvist and Sjöström (1969), who did not see any rise of the basal outflow of NA from the isolated spleen in the presence of PBA.

The unchanged levels of ATP and ^{45}S -labelled compounds in the noradrenergic vesicle fractions after administration of the drugs indicate that the drugs do not affect these vesicle components.

The 50–55% reduction of particle-bound NA after stimulation of the splenic nerve with 10 Hz for 30 min is fully comparable with a 39% reduction of NA in PBA treated perfused spleen after stimulation with 1–10 Hz for 10–20 min (Hedqvist and Sjöström 1969). On the other hand, in the absence of drugs Euler and Hellner Björkman (1955) found no consistent changes in NA contents of the stimulated portion of the spleen in comparison to the unstimulated. It seems that synthesis and reuptake of NA allow the stores of NA to be maintained at approximately constant levels. Chubb *et al.* (1972), stimulating the nerve with 10 Hz for 30 min, did find a reduction of NA of about 27% even in the absence of drugs, but they compared spleens of different cats. As pointed out above, the comparison of spleens of different cats might well give misleading results, especially when the NA/protein ratio is used for evaluating the amount of NA in the whole spleen.

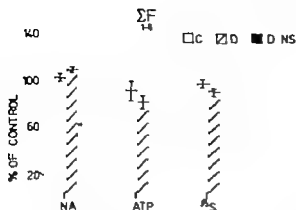


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Table IV Comparison of the effect of drug treatment on the total and non-lipid ^{35}S in the Flood gradient fractions. Cats were injected i.v. with $\text{Na}_2^{35}\text{SO}_4$ and i. with PBA (10 mg/kg) and imipramine (5 mg/kg) 24 h and 2 h before removal of the spleen, respectively. Particulate fractions of the spleen homogenates were isolated, and total and lipid-bound ^{35}S determined as described in Methods. Non-lipid ^{35}S , total ^{35}S , lipid-bound ^{35}S , ^{35}S per mg protein in each fraction from the dorsal portion is expressed as percentage of the corresponding value from the control frontal portion. Means \pm S.E. are given. The effect on total and non-lipid ^{35}S was correlated $r=0.79$, significant at $p=0.01$

	F1	F2	F3	F4	F5	F6
Total ^{35}S	104.1	83.3	98.8	98.1	76.4	80.5
% of control	54	± 3.1	± 6.7	± 7.5	± 7.3	± 5.1
Non-lipid ^{35}S	104.9	78.9	94.3	83.6	78.6	81.6
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While in our experiments nerve stimulation induced a 50% decrease in NA in noradrenergic vesicle fractions, it did not affect their contents of ATP indicating that substantial loss of ATP from the noradrenergic vesicles could have occurred. Also a significant decrease of the NA/ATP molar ratios in fractions 1-4 induced by nerve stimulation supports the contention that NA and ATP are not released together. Several observations by others favour this conclusion, too. Stjärne *et al.* (1970) accordingly found no increase in the outflow of ^3P labelled material from the spleen during stimulation and, simultaneously, no decrease of ATP in the splenic capsule, while 50% of the NA content of the spleen was released. Potter and Axelrod (1963) observed that a reserpine-induced reduction of NA in rat hearts was not accompanied by a fall of ATP. A decrease in ATP content of adipose tissue induced by nerve stimulation could be prevented by an α -receptor blockade which eliminated the vasoconstrictor effect of nerve stimulation and thereby the local hypoxia (Fredholm *et al.* 1977). The drop and subsequent increase of ATP in seminal duct after sympathetic denervation was considered to be mainly due to changes in effector cells, i.e. in the smooth muscle energy store (Wesfall *et al.* 1975).

The NA/ATP ratios of our controls (1.7-4.8) were in the range of similarly purified noradrenergic vesicle fractions from peripheral tissues (Potter and Axelrod 1963 2.9-4.1; Livett 1968 1.3-3.7). However, in vesicle preparations from the bovine splenic nerve trunk that were virtually free from ATP-containing contamination, the ratios were 6.8 ± 1 (Yen *et al.* 1976). Moreover, by extrapolation to *in vivo* conditions Lagercrantz *et al.* (1975) estimated the ratio to be 19 in the distal end of the splenic nerve and up to 10 in the nerve terminals. They therefore concluded that a major part of NA in the terminal is not bound to ATP. Consequently, one cannot expect it to be released together with ATP in a stoichiometric ratio (4:1). On the other hand, it has been well established that ATP is a powerful mediator of NA uptake (for references see Euler 1970) and may be expected to function as such also during the process of reuptake. Recent studies on isolated nerve vesicles support this view (Yen *et al.* 1973) since the rate of NA release from the vesicles is retarded in the presence of ATP: the reason for the slower net loss of NA might be an ATP-facilitated uptake. In addition, ATP itself was utilized at a much slower rate than NA was released (Yen *et al.* 1976). In view of these findings one might speculate that the ATP in noradrenergic vesicles, instead of being an inert binding substance which passively flows out of the terminal together with NA, has a more dynamic function. Such a function could be the utilization of ATP during the energy requiring process of reuptake that helps to maintain the transmitter economy at the nerve terminal. The blockade of the NA reuptake both in our own and in the experiments of Stjärne *et al.* (1970) would, of course, prevent such a utilization of ATP. Hence, the ATP levels will not change.

The non-lipid compounds labelled *in vivo* with ^{35}S -sulphate are presumably SMPSs and possibly sulphated glycoproteins (Robinson and Green 1962; Margolis and Margolis 1970). SMPSs have been identified in highly purified noradrenergic vesicles from bovine splenic nerves as well as in noradrenergic vesicle fractions from various sympathetically innervated tissues (Blaschke *et al.* 1976). Since drug treatment and nerve stimulation do not diminish the contents of ^{35}S -labelled compounds in any fraction, a significant loss of SMPSs during the release of NA might be excluded.

In conclusion: While stimulation of the splenic nerve was followed by a marked decrease of NA contents in the noradrenergic vesicle fractions, the contents of ATP and ^{35}S -SMPSs were not lowered. The finding is highly suggestive as an argument against the conjecture of complete exocytosis, i.e. the release of the entire soluble contents of the NA vesicle. Exocytosis has been defined as a process consisting of a fusion of the vesicular membrane with the plasmalemma, followed by emptying of the soluble contents of the vesicle directly into the exterior of the cell, without passing the cytoplasm (Katz 1969; Pfenninger 1973). While some authors imply that by exocytosis the entire soluble contents run out (Smith 1971; De Potter 1973), others consider the possibility that only a part of the contents come out by a so-called partial exocytosis (Geffen *et al.* 1970; Stjärne 1976).

Only a fraction of the soluble vesicle proteins is released together with NA during nerve stimulation (Geffen *et al.* 1970; Smith *et al.* 1970; Weismüllsboum *et al.* 1971; Gewirtz and Leyt 1970). Moreover the major mechanism for the "short-term" maintenance of NA in nerve terminals is an almost complete reuptake of the liberated NA (Hedqvist and Stjärne 1969). Such highly economic behaviour of the neuron is difficult to reconcile with a continuous loss of the total soluble part of the NA vesicle unless we assume that there is a reuptake of the macromolecular compounds. Our results are compatible with the concept of a partial exocytosis with a selective release of the transmitter. ATP may be utilized in the process of NA reuptake.

The mechanism of the ATP-independent storage and release of NA remains unclear. Lüscher (1973) speculated upon the existence of a functional transmitter pool in the nerve terminal vesicles with properties of a cation exchanger. The nerve impulse-induced transmitter release should be due to a cation exchange between the NA in the functional transmitter pool and inorganic ions in the neuro-effector junctional gap. The ion exchange should occur at the nerve terminal-vesicle contact area during the period of nerve impulse related depolarization. In analogy to the heparin-protein complex in mast cell granules (see Uvnäs 1977), a SMPS-protein complex was suggested as a possible matrix for a functional release pool in noradrenergic vesicles (Åborg *et al.* 1972; Blaschke *et al.* 1976). The present finding that SMPSs are not released together with NA on splenic nerve stimulation is in agreement with this hypothesis. Further insight into the mechanism of the ATP-independent storage and release of NA at the nerve terminals might emerge from studies presently going on in our laboratory.

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References

- Åborg, C.-H., G. FELLÖW, R. NORRÅL and B. UVNÄS, A sulphomucopolysaccharide-protein complex in the adrenergic vesicle (granule) fraction from nerves and tissues. *Acta physiol. scand.* 1972, **86**, 427-429.
- BLASCHKE, M. A. and M. FELLÖW, The storage of endogenous noradrenaline in sympathetic nerve terminals. *J. Physiol. (Lond.)* 1971, **215**, 163-179.
- BLASCHKE, M. A., U. HEDQVIST and B. UVNÄS, Identification of the mucopolysaccharides in catecholamine-containing subcellular particle fractions from various rat, cat and ox tissues. *Acta physiol. scand.* 1976, **114**, 120.

- CHURR, I. W. W. P. DE POTTER and A. F. DE SCHAEFFDYVER, Evidence for two types of noradrenaline storage particles in dog spleen. *Nature (Lond.)* 1970. 228 1703-1704.
- CHURR, I. W. W. P. DE POTTER and A. F. DE SCHAEFFDYVER, Influence of nerve stimulation on subcellular distribution of dopamine- β -hydroxylase in the dog spleen. *Experientia (Basel)* 1972. 293-295.
- COPPERSTEIN, S. J. and A. LAZAROW, A microspectrophotometric method for the determination of cytochrome oxidase. *J. biol. Chem.* 1951. 189 665-670.
- DEARNALEY, D. P. and L. B. GEFFEN, Effect of nerve stimulation on the noradrenaline content of the spleen. *Proc. R. Soc. B* 1966. 166 303-315.
- DE POTTER, W. P., Release of amines from sympathetic nerves. *Frontiers in Catecholamine Research* (eds. Usdin, E. and S. H. Snyder) Pergamon Press 1973. 471-475.
- DE POTTER, W. P., I. W. CHURR and A. F. DE SCHAEFFDYVER, Pharmacological aspects of peripheral noradrenergic transmission. *Arch. Internat. Pharmacodyn. Therapie* 1972. 195 (Suppl.) 54-287.
- DONTAS, A. S. and M. NICKERSON, Central and peripheral components of the action of ganglionic blocking agents. *J. Pharmacol. exp. Ther.* 1957. 110 147-159.
- EULER, U. S. VON, Effect of some metabolic factors and drugs on uptake and release of catecholamines in vitro and in vivo. In *New Aspects of Storage and Release Mechanisms of Catecholamines* (H. J. Schümann and G. Kroneberg, eds.) Bayer Symposium II Springer Verlag 1970. 144-158.
- EULER, U. S. VON and S. HELLMER-BÄCKEMAN, Effect of increased adrenergic nerve activity on the content of noradrenaline and adrenaline in cat organs. *Acta physiol. scand.* 1955. 33 (Suppl. 118) 17-22.
- FILLENZ, M. and P. R. C. HOWE, The contribution of small and large vesicles to noradrenaline release. *J. Physiol. (Lond.)* 1971. 212 4-43P.
- FILLENZ, M. and P. R. C. HOWE, Depletion of noradrenaline stores in sympathetic nerve terminals. *Neurochem.* 1975. 24 683-688.
- FOLKOW, B., J. HÄGGGREN and B. LEANDER, Extent of release and effluxion of noradrenaline peripheral adrenergic nerve terminals. *Acta physiol. scand.* 1967. 72 (Suppl. 307) 6-18.
- FREDHOLM, B., E. BRILFRAGEN and E. BLASCHKE, Changes in ATP and cyclic nucleotide levels during sympathetic nerve stimulation in canine subcutaneous adipose tissue in situ. *Acta physiol. scand.* 1971. 99 313-322.
- FURCHGOTT, R. F. and T. DE GUBAREFF, The determination of inorganic phosphate and creatine phosphate in tissue extracts. *J. biol. Chem.* 1956. 223 377-382.
- GEFFEN, L. B., B. G. LIVETT and R. A. RUSH, Immunohistochemical localization of chromogranins in sheep sympathetic neurons and their release by nerve impulses. In *New Aspects of Storage and Release Mechanisms of Catecholamines*. Eds. H. J. Schümann and G. Kroneberg. 1970. Bayer Symp. II. 56-7.
- GEWIRTZ, G. P. and I. J. KOPPEL, Release of dopamine- β -hydroxylase with norepinephrine during spleen nerve stimulation. *Nature (Lond.)* 1970. 227 406-407.
- GREEN, R. D. and W. W. FLEMING, Analysis of supersensitivity in the isolated spleen of the cat. *Pharmacol. exp. Ther.* 1968. 162 234-264.
- HEDQVIST, P. and L. STILLY, The relative role of capture and of de novo synthesis for the maintenance of neurotransmitter homeostasis in noradrenergic nerves. *Acta physiol. scand.* 1969. 75 270-281.
- HUBBARD, J. I., Mechanism of transmitter release. *Prager Symp. s. Molec. Biol.* 1970. 21 33-14.
- JØRSEN, L. L., *The uptake and storage of noradrenaline in sympathetic nerves*. Cambridge University Press 1967. Chapter 8.
- KATZ, B., *The release of neural transmitter substances*. Liverpool University Press 1969.
- LÄGERCRANTZ, H., Lipids of the sympathetic nerve trunk vesicles. Comparison with dendro-medullary vesicles. *Acta physiol. scand.* 1971. 82. 567-570.
- LÄGERCRANTZ, H., On the composition and function of large dense cored vesicles in sympathetic nerve. *Neuroscience* 1976. 1 81-92.
- LÄGERCRANTZ, H., G. FURUD and I. DAHLIN, An attempt to estimate the in vivo concentrations of noradrenaline and ATP in synaptic large dense cored nerve vesicles. *Acta physiol. scand.* 1975. 99. 136-138.
- LIVETT, B. G., *Site of noradrenaline synthesis in sympathetic neurons*. Thesis. Victoria, Australia, Monash University 1968.
- LOWRY, D. H., N. L. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951. 193 265-275.
- MARGOLIS, R. J. and R. U. MARGOLIS, Sulfated glycopeptides from rat glycoproteins. *Biochemistry* 1970. 9 4389-4396.

- NEED, D. L. and P. R. MOLLICHY, Distribution and properties of adrenergic storage vesicles in nerve terminals. *J. Pharmacol. exp. Ther.* 1976. 196. 344-359.
- ROBINSON, M. Non-equilibrium drug antagonism. *Pharmacol. Rev.* 1957. 9. 244-259.
- ROSENBLUM, K. H. *Synaptic morphology and cytochemistry*. G. Fischer Verlag, Stuttgart 1973. pp. 47.
- PUTER, L. T. and J. AXELSON, Properties of noradrenergic storage particles of the rat heart. *J. Pharmacol. exp. Ther.* 1963. 142. 299-305.
- ROSEN, J. D., JR. and J. P. CHEN, Sulphoamino polysaccharides in brain. *J. Biol. Med.* 1962. 35. 243-258.
- REIS, A. D., Summing up some implications of the neuron as a secreting cell. *Phil. Trans. R.* 1971. 261. 423-471.
- REIS, A. D., W. P. DE PUTTER, E. I. MORMAN and A. F. DE SCHAEPMEYER, Release of dopamine- β -hydroxylase and chromogranin A upon stimulation of the splenic nerve. *Time Cytol.* 1970. 2. 547-562.
- ROBERT, P. E. and S. E. WILLIAMS, Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Analyt. Biochem.* 1969. 29. 381-392.
- ROSEN, L., Basic mechanisms and local feedback control of secretion of adrenergic and cholinergic neurotransmitters. In *Handbook of Psychopharmacol.* 1976. Vol. 6. 179-233.
- ROSEN, L., P. HEDQVIST and H. LAGERCRANTZ, Catecholamines and adenosine nucleotide material in efflux from stimulated adrenal medulla and spleen. *Biochem. Pharmacol.* 1970. 18. 1147-1152.
- ROSEN, M. A. Glucose-6-phosphatase from liver. In Colowick, S. P. and M. O. Kaplan (eds.), *Methods Enzymology* 1955. Vol. 11. 541-543. Academic Press, N.Y.
- TELFORD, J. B. New aspects of the localization of catecholamines in adrenergic neurons. In E. Ueda and S. H. Snyder (eds.), *Frontiers in Catecholamine Research*. Pergamon Press 1973. 433-438.
- DEUTSCHER, S. Fluorescence assay in biology and medicine. Academic Press 1962. Pp. 139.
- UEDA, E., The mast cell and its biogenic amines. Proc. 9th Int. Congr. Pharmacology San Francisco 1972. In *Pharmacology and the Future of Man* 1972. 4. 304-326.
- UEDA, E. An attempt to explain nervous transmitter release as due to nerve impulses induced cation exchange. *Acta physiol. scand.* 1973. 87. 168-175.
- UEDA, E. The mechanism of histamine release from mast cells. In *Handbook of exp. Pharmacol.* 1977. Vol. XVIII/2. Springer Verlag Berlin-Heidelberg.
- UEDA, E. and C.-H. ÅNGER, The ability of ATP-free granule material from bovine adrenal medulla to bind inorganic cation and biogenic amines. *Acta physiol. scand.* 1977. 99. 476-483.
- WATKINS, R. M., N. B. THOA, D. O. JOHNSON, J. J. KOVICH and J. AXELSON, Proportional release of norepinephrine and dopamine- β -hydroxylase from sympathetic nerves. *Science* 1971. 174. 1349-1351.
- WATKINS, D. P., T. F. LI and R. E. SUTHER, Morphological and biochemical changes in supercontractile smooth muscle. *Fed. Proc.* 1975. 34. 1963.
- YU, R. L., R. L. KLEY and S. H. CHEN-YEN, Highly purified splenic nerve vesicles, early post-mortem effects on the noradrenergic contents and pools. *J. Neurocytol.* 1973. 2. 1-12.
- YU, R. L., R. L. KLEY, S. H. CHEN-YEN and A. THURBERG-KLEY, Noradrenergic-adenosine triphosphate ratio in purified adrenergic vesicles. *J. Neurobiol.* 1976. 7. 11-22.

- CIRUŠ, I. W. W. P. DE POTTER and A. F. DE SCHAEFFDRYVER, Evidence for two types of noradrenaline storage particles in dog spleen. *Nature* (Lond.) 1970, 228, 1203-1204.
- CIRUŠ, I. W. W. P. DE POTTER and A. F. DE SCHAEFFDRYVER, Influence of nerve stimulation on the subcellular distribution of dopamine- β -hydroxylase in the dog spleen. *Experientia* (Basel) 1972, 28, 293-295.
- COPPERSTEIN, S. J. and A. LAZAROW, A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* 1951, 189, 665-670.
- DIARMALPY, D. B. and L. B. GIFFEN, Effect of nerve stimulation on the noradrenaline content of the spleen. *Proc. R. Soc. B* 1966, 166, 303-315.
- DE POTTER, W. P. Release of amines from sympathetic nerves. *Frontiers in Catecholamine Research* (eds. Uddin, L. and S. H. Snyder), Pergamon Press 1973, 471-475.
- DE POTTER, W. P., I. W. CIRUŠ and A. F. DE SCHAEFFDRYVER, Pharmacological aspects of peripheral noradrenergic transmission. *Arch. Internat. Pharmacodyn. Therapie* 1972, 196, (Suppl.) 258-287.
- DONTAS, A. S. and M. NICKERSON, Central and peripheral components of the action of ganglionic blocking agents. *J. Pharmacol. exp. Ther.* 1957, 120, 147-159.
- EULER, U. S. von, Effect of some metabolic factors and drugs on uptake and release of catecholamines in vitro and in vivo. In *Aspects of Storage and Release Mechanism of Catecholamines* (H. J. Schümann and G. Kroneberg, eds.), Bayer Symposium II Springer Verlag 1970, 144-158.
- EULER, U. S. von and S. HELLING-BJÖRKMAN, Effect of increased adrenergic nerve activity on the content of noradrenaline and adrenaline in cat organs. *Acta physiol. scand.* 1955, 33, Suppl. 118, 17-20.
- FELLENZ, M. and P. R. C. HOWE, The contribution of small and large vesicles to noradrenaline release. *J. Physiol.* (Lond.) 1971, 212, 42-43P.
- FELLENZ, M. and P. R. C. HOWE, Depletion of noradrenaline stores in sympathetic nerve terminals. *J. Neurochem.* 1973, 24, 683-688.
- FOLKOW, B., J. HJØGENDAL and H. LINDER, Extent of release and elimination of noradrenaline in peripheral adrenergic nerve terminals. *Acta physiol. scand.* 1967, 72, Suppl. 307, 6-38.
- FREDHOLM, B., E. BELFRADE and E. BLASCHKE, Changes in ATP and cyclic nucleotide levels during sympathetic nerve stimulation in canine subcutaneous adipose tissue in situ. *Acta physiol. scand.* 1977, 99, 313-322.
- FURCHGOTT, R. F. and T. DE GUARRETT, The determination of inorganic phosphate and creatine phosphate in tissue extracts. *J. Biol. Chem.* 1936, 223, 377-388.
- GIFFEN, L. B., B. G. LIVETT and R. A. RUSH, Immunohistochemical localization of chromogranins in sheep sympathetic neurons and their release by nerve impulses. In *New Aspects of Storage and Release Mechanisms of Catecholamines*, Eds. H. J. Schümann and G. Kroneberg, 1970, Bayer-Symp. II, 58-72.
- GEWIRTZ, G. P. and I. J. KOPPEL, Release of dopamine- β -hydroxylase with norepinephrine during spleen nerve stimulation. *Nature* (Lond.) 1970, 227, 406-407.
- GREEN, R. D. and W. W. FLEMING, Analysis of supersensitivity in the isolated spleen of the cat. *J. Pharmacol. exp. Ther.* 1968, 162, 254-264.
- HEDQVIST, P. and L. STJÄRNE, The relative role of recapture and of de novo synthesis for the maintenance of neurotransmitter homeostasis in noradrenergic nerves. *Acta physiol. scand.* 1969, 76, 270-283.
- HUBBARD, J. I., Mechanism of transmitter release. *Progr. Biophys. & Molec. Biol.* 1970, 21, 33-154.
- IVERSEN, L. L., *The spleen and storage of noradrenaline in sympathetic nerves*, Cambridge University Press, 1967, Chapter 8.
- KATZ, B., *The release of neural transmitter substance*, Liverpool University Press 1969.
- LAGERCRANTZ, H., Lipids of the sympathetic nerve terminal vesicles. Comparison with adrenal-medullary vesicles. *Acta physiol. scand.* 1971, 82, 567-570.
- LAGERCRANTZ, H., On the composition and function of large dense cored vesicles in sympathetic nerves. *Neuroscience* 1976, 1, 81-92.
- LAGERCRANTZ, H., O. FRID and I. DAHLIN, A. attempt to estimate the in situ concentrations of noradrenaline and ATP in synaptic large dense cored nerve vesicles. *Acta physiol. scand.* 1975, 94, 136-138.
- LIVETT, B. O., *Study of noradrenaline synthesis in sympathetic neurons*, Thesis, Victoria, Australia, Monash University 1968.
- LOWRY, O. H., N. I. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951, 193, 265-275.
- MARQUEL, R. K. and R. U. MARQUEL, Sulfated glycopeptides from rat glycoproteins. *Biochemistry* 1970, 9, 4389-4396.

non increase the level of tissue cadaverine and such an effect might influence its synthesis. The diamine oxidase activity is known to be high in the placenta and plasma of pregnant rats (Roberts and Robson 1953, Kobayashi 1964).

Methods

Adult female rats, weight 140-180 g, of the Sprague-Dawley strain are used. They were fed standard chow and water ad libitum. Their estrous cycles were followed by vaginal cytology. Smears were taken in the morning and stained with methylene blue. Only rats with normal estrous cycles are used. Rats which were found to become pregnant were allowed to mate in the pro-estrous or early estrous phase. They were mated for 24 h. The next morning when sperm was found in the vaginal smear was considered as the first day of pregnancy. On the 19th day of pregnancy the rats were killed by cervical dislocation and eviscerated. Concomitantly control rats are sacrificed. One group of rats was studied under unaltered conditions and another group after the injection of the diamine oxidase inhibitor minipopsadine (70 mg minipopsadine hemisulphate/kg) for 10 consecutive days before the actual experiment.

Tissues of pregnant rats (ovary, uterus, kidney, liver, fetus and placenta) and of non-pregnant rats (liver, uterus, kidney and liver) were rapidly removed, cooled on ice, minced with scissors and gently homogenized in Dounce type homogenizer in 4 volumes of cold 0.1 M sodium phosphate buffer (pH 7.2) containing 5×10^{-4} M dithiothreitol, 10^{-4} M EDTA and 2% (w/v) glucose. The homogenate was centrifuged at 2000 g for 20 min at 4°C. The supernatant is used as enzyme source. Supernatant corresponding to 10 mg of tissue (ovary, kidney, liver and fetus), 40 mg of tissue (placenta in its whole, maternal and fetal lobes) and 16-57 mg (ovary) was incubated in the presence of pyridoxal-5-phosphate (final conc. 10^{-4} M), DL-(1- 14 C)-lysine monohydrochloride (final conc. 10^{-6} M, sp. act. 10 μ Ci/mole) and the same buffer as used for homogenization, the total finally made up to 1.0 ml. The incubation was carried out at 37°C for 60 min with agitation. The reaction was stopped by the tipping of 0.7 ml of 2 M citric acid from the side arm of the incubation vessel. Expelled 14 CO₂ was trapped on 10 mm piece of No. 005 'Mallinckrodt' filter paper prepared with 100 μ l hydrazine of Hyamine (1 M solution in methanol). For maximal efficiency of the 14 CO₂ the shaking was continued for another 45 min. After completed incubation the filter paper was dropped into acidification vessel containing 8 ml of acidification solution (Bray 1960) and its radioactivity measured in Packard Tri-Carb liquid scintillation spectrometer.

For enzymatic determinations of cadaverine formation experiments of the placenta and ovary were incubated in the presence of minipopsadine to final concentration of 2×10^{-4} M to prevent oxidative decarboxylation of the formed cadaverine by diamine oxidase. The reaction was stopped by the tipping of 0.7 ml of citric acid from the side arm of the incubation vessel. The amount of 14 CO₂ expelled was compared with the amount of the cadaverine formed in the same incubate. For the preparation of the sample for analysis of its cadaverine content 0.4 mg EDTA was added to each ml of the incubate. The samples were then centrifuged at 800 g for 10 min whereafter the samples were adjusted to pH values of 2.8-2.9 and filtered through filter with pore size of 0.22 μ m (Mallinckrodt Corp.). The separation and quantitative estimation of cadaverine in the incubates were carried out on 6-78 mm column of Duroon SC-44, using an automatic amine acid analyzer (LKB-BIOLAB, 3201). The procedure as described by Anderson (1978) was used with minor modifications.

For determination of the content of cadaverine in the tissues of pregnant and non-pregnant rats about 20 mg of tissue was homogenized in Dounce type homogenizer in 3.5 ml sulfolactic acid (40 mg sulfolactic acid and 0.4 mg EDTA/ml). The extracts were then boiled on water-bath for 30 min, whereafter they were prepared and assayed in the same way as described above for determination of cadaverine content in incubates.

Results

Cadaverine formation in various tissues from pregnant and non-pregnant rats

The formation of cadaverine in vitro in various tissues from pregnant rats was examined at first, then, from an earlier investigation (Anderson *et al.* 1978), the urinary excretion of

Formation of cadaverine in the pregnant rat

By

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Abstract

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The formation as well as the content of cadaverine were determined in different tissues of pregnant and non-pregnant rats. The placenta and ovary were most potent in the ability to form cadaverine. To our knowledge this is the first report of an *in vitro* formation of cadaverine linked to a normal physiological process, i.e. pregnancy. The highest concentration of cadaverine was found in the placenta and ovary of the pregnant rat. Treatment with aminoguanidine generally elevated the content of cadaverine, indicating a role of diamine oxidase as a regulator of diamine content.

Key words: Cadaverine formation, content of cadaverine, rat pregnancy

Evidence of *in vitro* formation of cadaverine in mammalian tissue was originally reported in the mouse kidney after the injection of the anabolic steroid Durabolin (Henningsson, Persson and Rosengren 1976). An *in vivo* formation of cadaverine is supported by the observation that rats regularly excrete cadaverine in the urine. The amine has also been detected in human urine (Bremer and Kohne 1971). In the pregnant rat the urinary excretion of cadaverine together with other diamines as well as the polyamine spermidine is greatly increased (Andersson, Henningsson and Rosengren 1978). On the 15th day of pregnancy the cadaverine excretion began to rise and hence increased continuously to day 19-20 of pregnancy when peak values up to 10 times the non-pregnant level were observed. Thereafter the cadaverine excretion fell towards the non-pregnant level which after the birth of the young was resumed.

The purpose of the present study was to investigate in which tissue(s) of the pregnant rat an increased formation of cadaverine takes place. In addition the content of cadaverine was determined in various tissues of the pregnant and non-pregnant rat. As stated above, very little is known about the synthesis of cadaverine in mammalian tissues. Regarding the close congener of cadaverine, i.e. putrescine, it has been shown that its synthesis is controlled by the level of the amine itself in different cell culture systems and in intact animals (for review see Jänne, Pökö and Raina 1978). The diamine oxidase inhibitor aminoguanidine might a

Table II. Content of cadaverine (nmol/g) in different tissues of pregnant and non-pregnant rats. The means of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values \pm S.E. of mean of 4 determinations, except for those indicated with (a) which stands for 3 determinations. A, untreated rats, B, rats treated with aminoguanidine. II stands for not measurable amount.

Tissue	Pregnant		Non-pregnant
	A	B	B
Ovary	14 \pm 4.75	28 \pm 5.04	4.1 \pm 1.62
Uterus	3.9 \pm 2.44	5.3 \pm 1.78	2.7 \pm 1.96
Salivary	0	23 \pm 3.10	32 \pm 2.71
Liver	7.6 \pm 3.00	14 \pm 4.22	7.9 \pm 0.43
Testis	3.3 \pm 3.25	11 \pm 1.61 ()	
Whole placenta	10 \pm 1.43	14 \pm 2.42	
Fetal placenta	13 \pm 0.88	15 \pm 1.20 (a)	
Maternal placenta	9.5 \pm 2.05	20 \pm 3.28 (a)	

$p < 0.05$ and $p < 0.01$ versus pregnant rats treated with aminoguanidine.

Parallel assays of $^{14}\text{CO}_2$ -production and the formation of cadaverine from DL-(1- ^{14}C)-lysine are performed to ensure that the evolution of ^{14}CO provided a valid measure of the biosynthesis of cadaverine. Values of cadaverine formation in the placenta and the ovary are shown in Table II from which it is apparent that there was a fair accordance in results obtained by the two methods showing that artifactual release of ^{14}CO did not occur.

Content of cadaverine in various tissues from pregnant and non-pregnant rats

The cadaverine concentration in tissues of pregnant rats was determined. Of these rats one group had been injected with aminoguanidine for 10 days before the actual experiment, the other group was untreated. The results are summarized in Table III in which also results from non-pregnant rats given aminoguanidine are presented. In the untreated pregnant rat most cadaverine was found in the ovary and placenta. Aminoguanidine treatment of the animals generally elevated the cadaverine concentration of the tissues. The most distinct increase was seen in the kidney an organ known to be a source of diamine residues. Also in the non-pregnant group the highest concentration of cadaverine was found in the kidney. Notable is the significant difference in the cadaverine content of ovary between the pregnant and non-pregnant animals, a result which might reflect the high synthesis in the ovary of the pregnant rat.

Discussion

Although it has been known for some time that cadaverine is regularly excreted in the urine its origin is uncertain. Most authors regard the amine as a contaminant believed to be formed by bacteria in the lumen of the intestine, absorbed and carried by the blood to tissues and urine. However some recent findings strongly suggest that cadaverine can be synthesized in mammalian tissues both in vivo and in vitro. Cadaverine has been found in the brain and blood of germ-free mice (Stepha-Klauco and Dolzalova 1974). An in vitro formation of cadaverine in mouse kidney has been observed after the injection of the anabolic steroid

TABLE I Formation of cadaverine (nmol/g \times h) in different tissues of pregnant and non-pregnant rat. The tissues of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values \pm S.E. of mean of 8 observations (tissues from 4 untreated rats and 4 rats treated with aminoguanidine)

Tissue	Non-pregnant	Pregnant
Ovary	25 \pm 7.47	110 \pm 16.3
Uterus	2. \pm 3.40	9.0 \pm 1.49
Kidney	2.1 \pm 1.03	3.9 \pm 1.38
Liver	2.7 \pm 2.11	1.8 \pm 1.01
Fetus		5.9 \pm 1.94
Whole placenta		250 \pm 20.9
Fetal placenta		350 \pm 29.0
Maternal placenta		5. \pm 10.0

= $p < 0.01$ and = $p < 0.001$ versus non-pregnant.

cadaverine is known to be high, *i.e.* on the 19th day of pregnancy. In addition, determinations were made on tissues of non-pregnant female rats. The tissues were collected from two groups of rats of which one group was treated with aminoguanidine as states above the other was not. Since no difference was found between the values of the two groups, the figures are brought together and presented in Table I from which it will be seen that of the tissues examined the placenta was most potent in the ability to form cadaverine, followed by the ovary and the uterus. As to the uterus the activity was higher in that of the non-pregnant rat than in that of the pregnant one. This may be attributed to the fact that the activity of cadaverine formation is expressed in nmol per g wet weight of the tissue. It is known that the pregnant uterus is edematous and for that reason the total activity of the organ may not be reduced during pregnancy. Both kidney and liver showed low cadaverine formation and no differences between pregnant and non-pregnant rats were observed.

The question arose as to which part of the placenta, the maternal one and/or the fetal one, contributed to the synthesis of cadaverine. For that reason the organ was macroscopically dissected and the enzyme activity of the maternal and fetal parts was determined. It was found that the enzyme responsible for the formation of cadaverine was mainly located in the fetal placenta, in fact the synthesis of cadaverine in the maternal part was only 15% of that in the fetal one (Table I).

TABLE II Stoichiometric determinations of cadaverine formation in tissues of the pregnant rat. Parallel assays of $^{14}\text{CO}_2$ production and formation of cadaverine (nmol/g \times h) were carried out in the same incubate.

	$^{14}\text{CO}_2$	Cadaverine
Whole placenta	340	340
	280	330
	220	180
	200	170
Fetal placenta	410	450
	340	440
Ovary	64	50
	43	39

TABLE II Content of cadaverine (nmol/g) in different tissues of pregnant and non-pregnant rats. The tissues of the pregnant rats were excised on the 19th day of pregnancy. The figures are mean values \pm S.E. of mean of 4 determinations, except for those indicated with (a) which stands for 3 determinations. A: untreated rats, B: rats treated with aminoguanidine. 0 stands for not measurable amount.

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Fetal placenta	13 \pm 0.89	15 \pm 1.20 (a)	
Maternal placenta	9.5 \pm 2.09	20 \pm 3.28 (a)	

* $p < 0.05$ and ** $p < 0.01$ versus pregnant rats treated with aminoguanidine.

Preliminary assays of $^{14}\text{CO}_2$ -production and the formation of cadaverine from DL-(1- ^{14}C)-lysine were performed to ensure that the evolution of ^{14}CO provided a valid measure of the biosynthesis of cadaverine. Values of cadaverine formation in the placenta and the ovary are shown in Table II from which it is apparent that there was a fair accordance in results obtained by the two methods showing that artifactual release of ^{14}CO did not occur.

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Durabolin (Henningsson *et al* 1976) In the present investigation an *in vitro* formation of cadaverine was linked to a normal physiological process, *i.e.* pregnancy

The high formation of cadaverine in the fetal part of the placenta is reflected in an elevated excretion of cadaverine during the last trimester of rat pregnancy (Andersson *et al* 1978) This circumstance is supported by experiments carried out on the 19th day of pregnancy showing that after the removal of the fetuses without dislodging the placenta the high urinary excretion of cadaverine was maintained, while on removing also the placenta the increased cadaverine excretion was abolished (to be published)

The elevated *in vitro* formation of cadaverine in the ovary during pregnancy is consistent with an increased endogenous cadaverine content of the organ indicating that cadaverine can be formed *in vivo* by decarboxylation of lysine.

Our present results together with our earlier findings of a regular pattern of urinary cadaverine excretion during pregnancy and the observation that cadaverine was formed in the mouse kidney stimulated to growth by an anabolic steroid strongly support our opinion that cadaverine is formed by mammalian tissue cells, and is not the product of bacterial decarboxylation of lysine

Aminoguanidine treatment of the animals doubled the concentration of cadaverine in the maternal part of the placenta which is very rich in diamine oxidase. In the fetal part with a low content of diamine oxidase the change in cadaverine content was only slight. It appears conceivable that diamine oxidase could function as a regulator of cadaverine content in certain tissues rich in the enzyme. Another role of diamine oxidase in the maternal part of the rat placenta may be to protect the maternal tissues from diamine derived from the fetuses and/or the fetal part of the placentas.

The results presented here support our view of a connection between diamine metabolism and the hormonal status prevailing in pregnancy and are also another example of elevated diamine formation in relation to growth.

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References

- ANDERSSON, A.-C., S. HENNINGSSON and E. ROSENQREN, Increased formation of diamines and polyamines in the pregnant rat. *J. Physiol. (Lond.)* 1978. 1 press.
- BRAY G. A., A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter *Analyt. Biochem.* 1960. 1 279-285
- BREMER, H. J. and E. KOFINA, The excretion of diamines in human urine. *Chin. chim. Acta* 1971 32 407-41
- HENNINGSSON, S., Automated liquid chromatography - research on histamine metabolism. *Agents and Actions* 1978 8 392-393
- HENNINGSSON, S., L. PERSSON and E. ROSENQREN, Biosynthesis of cadaverine in mice under the influence of an anabolic steroid. *Acta physiol. scand* 1976. 98 445-449
- JÄNNE, J., H. PÖSSÖ and A. RADNA, Polyamines in rapid growth and cancer. *Biochem. biophys. Acta* (Amst.) 1978 473 241-293.
- KORAYASHI, Y. Plasma diamine oxidase titres of normal and pregnant rats. *Nature (Lond.)* 1964. 201 146-147
- ROBERTS, M. and J. M. ROSSON, The histaminase content of the rat uterus and its relation to the decidua. *J. Physiol. (Lond.)* 1953. 119 286-291
- STEPHA KLAUCO, M. and H. DOLEZALOVA, Cadaverine in the brain of axenic mice. *Nature (Lond.)* 1974. 252 158-159

Prevention of protein extravasation in the brain by an anion transport inhibitor in acute experimental hypertension in rats

By

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Abstract

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Acute experimental hypertension induces protein leakage in the brain. The protein is thought to be, at least partly, transported through the endothelial cells by pinocytosis. An anion transport inhibitor (4-acetamido-4-isothiocyano-stilbene-2,2'-disulfonic acid, markedly reduced adrenaline or bradykinin-induced leakage of 125 I-FBSA and Evans blue-albumin in all areas of the rat brain. The preventive effect of bumetanide was less pronounced and no effect was seen of halopendol.

In vivo: Acute hypertension, blood brain barrier protein transport, pinocytosis, anion transport inhibitor bumetanide.

The metabolic and structural integrity of the endothelial cells of cerebral blood vessels is a prerequisite for the maintenance of the blood brain barrier (BBB) to macromolecules. Abrupt rise in arterial blood pressure can result in multifocal areas of protein extravasation. In addition to the degree and rapidity of the blood pressure increase extravasation can be modified by factors that influence the cerebral vascular tone. Vasodilatory anesthetics and drugs as well as hypercapnia increase the protein leakage whereas hypocapnia has a constrictive effect (Johansson 1976 a, b, Forster *et al.* 1977). Furthermore, some substances such as dexamethasone, reduce protein extravasation by a not clarified mechanism (Einarsson, Barlow and Lorenzo 1970, Johansson 1978).

The anion transport blocking drug 4-acetamido-4-isothiocyano-stilbene 2,2'-disulfonic acid (SITS) has recently been demonstrated to prevent the cholera toxin induced intestinal hypersecretion as well as cholera toxin induced activation of adenylyl cyclase (Lénnerroth, Hansson, Lange and Holmgren, in preparation). The secretion of serotonin from storage vesicles in human platelets by exocytosis is also inhibited by SITS (Pollard *et al.* 1977).

Previous studies have demonstrated that proteins extravasated in the brain after acute hypertension at least to a great extent are transported thorough the endothelial cells by

TABLE 1. Mean arterial pressure (MAP) before and after the blood pressure elevation induced by bicuculline or adrenaline. PaCO_2 immediate prior to the pressure rise. Mean value \pm S.E. PaO_2 was > 13.3 kPa (100 mmHg) in all rats.

Experimental group	n	Initial MAP mmHg	Maximum MAP	PaCO_2 , kPa
<i>Bicuculline</i> (1.0 mg kg^{-1})				
Controls	6	142 ± 3	187 ± 5	4.66 ± 0.03
SITS (25 mg kg^{-1})	6	136 ± 2	185 ± 2	4.92 ± 0.17
Dexamethasone (4 mg kg^{-1})	6	139 ± 3	196 ± 2	4.65 ± 0.09
Haloperidol (1 mg kg^{-1})	6	132 ± 4	189 ± 3	4.91 ± 0.13
<i>Adrenaline</i> ($20 \mu\text{g kg}^{-1}$)				
Controls	8	126 ± 6	194 ± 2	4.44 ± 0.10
SITS (25 mg kg^{-1})	8	134 ± 3	192 ± 2	5.00 ± 0.06

pinocytosis (Hansson, Johansson and Blomstrand 1975, Hansson and Johansson 1979, Nag, Robertson and Dinsdale 1977, Westergaard van Deurs and Brondsted 1977), *Le* process not normally occurring in cerebral blood vessels.

Transendothelial transfer of vesicles is energy dependent and could theoretically be influenced by SITS. The aim of the present study was to test if it was possible to prevent the protein extravasation in the brain caused by acute hypertension by pretreatment with SITS.

The blood pressure increase was induced either by adrenaline or by bicuculline. Bicuculline is a GABA receptor blocking agent that induces epileptic activity and hemodynamically gives rise to hypertension and a massive cerebral vasodilatation (Meldrum and Nilsson 1976). An extensive and exclusively pressure dependent BBB dysfunction is observed (Johansson and Nilsson 1977).

For comparison, some rats were pretreated with dexamethasone. It has recently been claimed that chlorpromazine decreases albumin extravasation around cerebral stab wounds (Rosengren *et al* 1978) and the authors postulated a possible effect via inhibition of the adenylate cyclase. In an earlier study we found a preventive effect of haloperidol in amphetamine induced BBB dysfunction which, however, in that study was thought to be secondary to an effect on the blood pressure (Carlsson and Johansson 1978). As haloperidol is closely related to chlorpromazine we included a trial with haloperidol.

Material and methods

Male Sprague-Dawley rats weighing 250–350 g were used. Anesthesia was induced with diethyl ether the rats tracheotomized, immobilized with suxamethonium chloride and mechanically ventilated with a gas mixture of 30% O_2 and 70% N_2O . Cannulae were inserted in one femoral vein (for administration of drugs) and in both femoral arteries (for electromanometric recording of mean arterial pressure, MAP and for sampling arterial blood for blood gas determinations). Body temperature was kept close to 37°C by means of intermittent external heating. Repeated blood gas determinations were made to ensure stable baseline of intermittent external heating. SITS (Nutritional Biochemical Co, Cleveland, Ohio, USA), 25 mg kg^{-1} or dexamethasone, 4 mg kg^{-1} was injected i.v. 60 min prior to the induction of hypertension. Haloperidol, 1 mg kg^{-1} was given i.v. 30 min before the hypertensive drug. For the number of rats in the various groups see Table 1. Before the blood pressure increase was induced, 2 ml of a 2

TABLE II 125 IHSA content in the brain 3 min after the administration of baclofine (1.2 mg kg^{-1}) in controls and in rats pretreated with SITTS, dexamethasone or haloperidol. The tracer content is expressed as 100 (counts $\text{mm}^{-2} \text{mg}^{-1}$ brain tissue)/(counts $\text{mm}^{-2} \text{mg}^{-1}$ blood). Mean values \pm S.E. ($n = 6$ in all groups).

Experimental group	Cerebral cortex	Thalamus	Cerebellum
Control	0.18 ± 0.03	0.94 ± 0.17	0.29 ± 0.03
SITTS (25 mg kg^{-1})	0.08 ± 0.01	0.22 ± 0.01	0.09 ± 0.02
Dexamethasone (4 mg kg^{-1})	0.11 ± 0.01	0.30 ± 0.13	0.21 ± 0.05
Haloperidol (1 mg kg^{-1})	0.15 ± 0.02	0.71 ± 0.08	0.23 ± 0.02

SITTS 4-acetamido-4-isobutyro-2-thiophene-2,2'-disulfonic acid disodium.
 $^*P < 0.01$ for differences from control rats.

areas of Evans blue in ml kg^{-1} (a index in vivo binds to serum albumin) and $100 \mu\text{Ci kg}^{-1}$ of 125 IHSA (low molecular weight) were injected 3 min after the administration of adrenaline, $20 \mu\text{g kg}^{-1}$ or baclofine, 1.2 mg kg^{-1} the brain as perfused in saline in situ for 1 min to rinse the cerebral vessels. The extravasated parts are subjected to scintillation counting and the extravasation of 125 IHSA is expressed as 100 (counts $\text{mm}^{-2} \text{mg}^{-1}$ brain tissue)/(counts $\text{mm}^{-2} \text{mg}^{-1}$ blood). The blood sample is taken immediately before starting of the perfusion through the heart.

Statistical differences are evaluated with Wilcoxon rank sum test.

Results

MAP before and after the pressure increase and PaCO_2 values immediately prior to the administration of baclofine or adrenaline are given in Table I. Macroscopically Evans blue-chromic extravasation was less in the SITTS treated groups and 125 IHSA leakage was especially less in all areas of the brain in rats given SITTS (Table II and III). This was particularly evident in rats given adrenaline in spite of higher PaCO_2 in the SITTS treated groups. Thus 7 out of 8 treated rats showed no or little extravasation and the IHSA ratio was $0.03-0.08$. (The tracer content in control brains from rats not subjected to hypertension is $0.1-0.4$ at the present technique of perfusion, see Carlsson and Johansson 1978). However as can be seen from the mean value and large standard error (Table III) no protective effect was seen in one rat in the SITTS-treated group. Compared to controls there was less extravasation also after pretreatment with dexamethasone but the decrease was significant only for the cerebral cortex (Table II). Haloperidol had no protective effect on protein leakage in the present experimental situation.

TABLE III 125 IHSA content in the brain 3 min after an injection of adrenaline ($20 \mu\text{g kg}^{-1}$) in controls and in rats pretreated with SITTS. The tracer content is expressed as 100 (counts $\text{mm}^{-2} \text{mg}^{-1}$ brain tissue)/(counts $\text{mm}^{-2} \text{mg}^{-1}$ blood). Mean values \pm S.E. ($n = 8$ in both groups).

Experimental group	Telencephalon	Diencephalon, mesencephalon	Pons, medulla oblongata	Cerebellum
Control				
SITTS (25 mg kg^{-1})	0.47 ± 0.13	0.36 ± 0.10	0.33 ± 0.07	1.16 ± 0.50
	0.18 ± 0.11	0.10 ± 0.03	0.10 ± 0.02	0.23 ± 0.13

SITTS 4-acetamido-4-isobutyro-2-thiophene-2,2'-disulfonic acid disodium.
 $^*P < 0.01$

Discussion

The results obtained in the present study demonstrate that SITS reduces the protein extravasation occurring in the brain after an abrupt increase of the blood pressure in rats. Theoretically SITS could either mediate a change in the cerebrovascular tone or could have a direct effect on the endothelial cell membrane. Although the first possibility cannot be excluded until hemodynamic studies have been performed an action on the cell membrane seems more likely.

In the present experimental models the extravasated proteins are probably mainly transported across the endothelial cells by pinocytosis (ref. see Introduction). Very few vesicles are observed in cerebral vessels under normal conditions (Reese and Karnovsky 1967). The wall distension induced by a rise in blood pressure seems to activate the endothelial cell membrane to form pinocytotic vesicles. It should be stressed that the formation and transendothelial transport of such vesicles are transitory phenomena in the present experimental models. When the blood pressure returns to normal levels the protein leakage ceases within 10–15 min (Petito, Schaefer and Plum 1977, Johansson and Linder 1978). Pinocytosis is an active process requiring activation of adenylyl cyclase (Marur *et al.* 1971, Joo, Rakonczay and Wollemann 1975). It has been demonstrated in another system that SITS prevents the activation of adenylyl cyclase induced by treatment of intestinal cells as well as of lymphocytes with cholera toxin (Lönnerth *et al.* in preparation). Furthermore, SITS inhibits the ATP-evoked epinephrine release from isolated chromaffin granules as well as the serotonin secretion from human platelets, induced by stimulation with thrombin or the calcium ionophore A23187 (Hoffman *et al.* 1976, Pollard *et al.* 1977), both processes being dependent upon adenylyl cyclase activation.

Obviously further studies are needed to evaluate the mechanisms behind the protective effect of SITS on pressure-induced BBB dysfunction. Work is in progress to study if SITS has any prophylactic effect on other types of BBB dysfunctions.

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References

- CARLSSON, C. and B. B. JOHANSSON, Blood brain barrier dysfunction after amphetamine administration in rats. *Acta neuropath. (Berl.)* 1978. 41: 125–129.
- ERDMERG, H. M., C. F. BARLOW and A. V. LORENZO, Effect of dexamethasone on altered brain vascular permeability. *Arch. Neurol.* 1970. 23: 18–22.
- FORSTER, A., K. VAN HORN, L. F. MARSHALL and H. M. SHAPIRO, Influence of anesthetic agents on blood brain barrier function during acute hypertension. *Acta neurol. scand.* 1977. 56, Suppl. 64: 60–61.
- HANSSON, H.-A. and B. B. JOHANSSON, Angiotensin-induced hypertension in rats. An ultrastructural comparison of brains from normotensive and spontaneously hypertensive rats. *Acta neuropath. (Berl.)* 1979. In press.
- HANSSON, H.-A., B. B. JOHANSSON and C. BLOMSTRAND, Ultrastructural studies on cerebrovascular permeability in acute hypertension. *Acta neuropath. (Berl.)* 1975. 32: 187–198.
- HOFFMAN, P. G., D. ZONDER, V. M. BONNER and H. B. POLLARD, Role of ATP and β - γ nucleotidyladenosine triphosphate in the stimulation of epinephrine protein release from isolated adrenal secretory vesicles. *Arch. Biochem.* 1976. 176: 375–383.

- JAISON, B. B. Brain barrier pathology in acute arterial hypertension. *Adv exp Med Biol* 1976 **a**, 69 37-57.
- JAISON, B. B. Some factors affecting the damaging effect of acute arterial hypertension on cerebral vessels. *Ann Clin Sci Med* 1976 **b**, 51 413-434.
- JAISON, B. B. Effect of dexamethasone on protein extravasation in the brain in acute hypertension induced by angiotensin. *Acta neuropathol* 1978, 57 180-185.
- JAISON, B. B. L. E. LINDER, Reversibility of the blood-brain barrier dysfunction induced by acute hypertension. *Acta neuropathol* 1978, 57 343-348.
- JAISON, B. B. and B. NELSON, The pathophysiology of the blood-brain barrier dysfunction induced by acute hypertension and by epileptic brain activity. *Acta neuropathol* (Berl.) 1977 **35** 153-158.
- JO, F. Z. RACONCELA M. WOLLEMAN, cAMP-mediated regulation of the permeability in the brain epithelium. *Experientia* (Basel) 1975, 31 582-584.
- KATO, S. K., E. HOLZMAN, L. L. SCHWARTZ and R. WALTER, Correlation between pinocytosis and intravascular adhesion by secretory phospholipase hormones and mediated by adenosine 3',5'-cyclic monophosphate. *J cell Biol* 1971, 49 522-534.
- NELSON, B. B. and B. JAISON, Cerebral blood flow and metabolic rate early and late in prolonged epileptic seizures induced in rats by bicuculline. *Brain* 1976, 99 523-542.
- JO, S. D. ROBERTSON and H. R. DUNN, Cerebral cortical changes in acute experimental hypertension. An ultrastructural study. *Lab Invest* 1977 **36**, 150-161.
- TOBI, C., J. A. SCHAEFER and P. PLUM, Ultrastructural characteristics of the brain and blood brain barrier in experimental seizures. *Brain Res* 1977 **127** 251-267.
- WILLIAMS, H. B. K. TACE-GOLDMAN, C. PADOVANI, C. E. CHUTEY and N. R. SHULMAN, Evidence for control of arylamine secretion from bovine platelets by hydroxyl ion transport and osmotic lysis. *Proc nat Acad Sci (Wash)* 1977, 74, 5295-5299.
- JO, T. S. and M. I. KARNOVSKY, Fine structural localization of blood-brain barrier to exogenous proteins. *J cell Biol* 1967 **34** 207-217.
- JAISON, B. B., L. L. PERSON and S. LAMON, Decrease of albumin extravasation slowed cerebral stab wounds after chlorpromazine treatment. *IRCS med sci* 1978, 6, 139.
- JAISON, B. B., AN DEITS, H. E. BRADSHAW, Increased vascular transfer of horseradish peroxidase from cerebral endothelium, evoked by acute hypertension. *Acta neuropathol* (Berl.) 1977 **37** 141-152.

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Paresthesiae. Abnormal impulse generation in sensory nerve fibres in man

By

H. ERIK TÖREBJÖRK, JOSÉ L. OCHOA and FRANCES V. MCCANN

The nature of the mechanisms underlying positive sensory symptoms, like spontaneous pain and paresthesiae, occurring in the setting of peripheral nerve dysfunction has remained speculative for powerful reasons. Indeed prerequisites to any investigation of that question include the ability to record, in alert human subjects, peripheral nerve signals that can be correlated with abnormal sensory experience and further the ability to analyse the pattern of activity in single afferent fibres of various types in relation to the spectrum of paresthetic sensations. Means to fulfill these requirements are now available. Using the microelectrographic technique of Vallbo and Hagbarth (1968) we have recorded peripheral nerve signals from single afferent fibres in awake subjects experiencing paresthesiae. Possible ethical reservations were circumvented by recording signals from our own peripheral nerves.

Post-ischemic paresthesiae were induced after release of a sphygmomanometer cuff that had remained inflated well above systolic blood pressure round the upper arm or the forearm for 15 to 40 min (Lewis *et al.* 1931, Zotterman 1933, Kugelberg 1944, Weddell and Sinclair 1947, Gilliatt and Wilson 1954, Nathan 1958). Neural signals were recorded with semi-microelectrodes inserted into sensory nerve fascicles of the median or the ulnar nerve proximal or distal to the site of the cuff. Details concerning signal recording, amplification, storage, and processing have been given elsewhere (Hagbarth *et al.* 1970). Fifteen experiments were performed on three healthy volunteers, and were not followed by complications of any kind.

Within 20 s of decompression and reestablishment of circulation to the cold numb hand a sensation of warmth rapidly leading to hot was perceived in the whole blushing hand. Approximately 40 to 60 s following release, high speed "buzzing" sensations of increasing intensity were referred predominantly to finger tips. Intensity peaked by 90 s. Striking spontaneous electrical discharges could be recorded during this period. Fig. 1 A shows the beginning of a characteristic high frequency discharge of relatively long duration (up to 7 s). Impulse frequency slowly decayed from an initial maximum as high as 240 imp/s. Recurring "buzzing" sensations and periods of sustained high frequency discharges were commonplace during this most intense stage of paresthesiae.

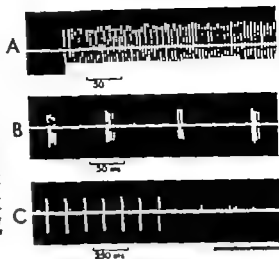


Fig. 1. Single unit impulses recorded in sensory nerve fascicles of the median nerve. A shows burst during periods of post-ischaemic paresthesia after release of cuff around the nerve. B and C represent activity in the same unit, but different from the unit in A. A: Abrupt beginning of high frequency discharges, lasting for several seconds, then subject reported sensations of prolonged "buzzing". B: Brief bursts of high frequency discharges repeated in rhythmic fashion. Subject felt sensations "tingling". C: Consistent of repeated double discharges was closely followed by subject's report of disappearance of long "buzzing" (burst).

As "buzzing" progressively diminished, distinct sensations described as intermittent "tingling" and short but painless pins and needles became the dominant feature between 90 and 120 s. Such sensations, which were also predominantly referred to finger tips, eventually substituted "buzzing" altogether. Pins and needles waned by the 4th minute, and then fading slowly decayed, to disappear after 10–20 min. Towards the end of this decrescendo period, it was often possible to identify discrete points of referral in the skin where the other, chaotic, intermittent paresthesiae, could be perceived individually in a rhythmic fashion. The neural activity recorded during intermittent paresthesia was dominated by brief bursts of high frequency discharges or by individual discharges sometimes repeated at various rhythms (Fig. 1 B). With time, the number of spikes per burst diminished and the interval between bursts increased, as the tingling sensation got fainter. On one occasion the subject deprived from audiovisual feedback reported disappearance of tingling referred to an identified field in the finger tip when the unit ceased to fire (Fig. 1 C).

These findings, based upon the application to man of techniques for single unit recording, confirm the prediction that paresthesiae from peripheral nerve dysfunction signify abnormal impulse generation in the sensory unit. Our studies indicate that recognizable changes in perception of paresthesiae (sustained buzzing versus intermittent "tingling") can be the consequence of modulation of impulse frequency and duration and rhythm of the discharges. Although the contribution to paresthesiae from different fibre types has not been investigated, the sustained, very high frequency of discharges recorded in some fibres endorses participation of myelinated fibres.

Studies are now in progress to help define the sites of abnormal impulse generation within the sensory unit, and to explore spontaneous activity in the spectrum of nerve fibre types during paresthesiae and neuritic pain.

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Post ischemic paresthesiae were induced after release of a sphygmomanometer cuff that had remained inflated well above systolic blood pressure round the upper arm or the fore arm for 15 to 40 min (Lewis *et al* 1931, Zotterman 1933, Kugelberg 1944, Weddell and Sinclair 1947, Gilliat and Wilson 1954, Nathan 1958). Neural signals were recorded with semi-microelectrodes inserted into sensory nerve fascicles of the median or the ulnar nerves proximal or distal to the site of the cuff. Details concerning signal recording, amplification, storage, and processing have been given elsewhere (Hagbarth *et al* 1970). Fifteen experiments were performed on three healthy volunteers, and were not followed by complications of any kind.

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Are there sensory neurons in the mucosa of the mammalian gut?

By

B. NEWSON, H. AHLMAN, A. DAHLSTRÖM, T. K. DAS GUPTA and L. M. NYHUS

The innervation of the gastrointestinal (G.I.) canal in mammals classically includes two plexi of ganglion cells, the submucous plexus and the myenteric plexus. Some nerve cells of these two plexi are probably of a sensory type (type II according to Dogiel 1896) and may send processes in the submucous-subglandular layer of the G.I. canal. However the existence of additional small nerve cells with elongated processes in the subglandular layer of the cow and pig stomach has been described (Vau 1932). During our studies on the morphological connection between nerves and epithelial cells of the small intestine of rat, we observed similar elongated nerve cells just beneath the crypt epithelium.

Male rats 150 to 200 g were decapitated. Small mucosal specimens were prepared from the ileum. The specimens were fixed in 4% buffered glutaraldehyde, postfixated in 2% OsO_4 , dehydrated, and embedded in Araldite. Ultrathin sections (600 Å) stained with lead citrate and uranyl acetate were observed under a transmission electron microscope (EMU 4 RCA).

In sections from the ileum, small, elongated cells with a light nucleus and floccular rough endoplasmic reticulum (rER) were sometimes observed just beneath the epithelium of the crypts. Their elongated processes were often separated from the crypt cells only by a basal lamina (Fig. 1). Membrane thickenings were occasionally seen on opposite sides of the separating lamina (Fig. 1 in). The processes, extending in parallel with the epithelium lining, mostly contained patches of rER, mitochondria, vesicles of varying sizes and electron-dense bodies, and occasionally microtubules. Other processes seemed devoid of rER but contained smooth ER tubules. This type of cell has the morphological characteristics of a nerve cell, and the extension of rER-containing processes (probably dendrites) may suggest that the neuron is of a sensory type. Support of the view that these cells are neurons is obtained from light microscopic immunohistochemical studies using Thy-1 antigens. The Thy-1 antigen has been shown to be present in large amounts in cell membranes of the thymus and neuronal elements in the rat, and appears to be specific for these two tissues in the adult rat (cf. Barclay and Hydén 1978). In the ileum Thy-1 positive cells were observed not only in the two nervous plexi of the gut wall, but also in elongated cells located just beneath the

References

- GILLIATT R. W. and T. G. WILSON, Ischaemic sensory loss in patients with peripheral nerve lesions. *J. Neurol. Neurosurg. Psychiat.* 1934 17 104-114.
- HAGBARTH, K. E., A. HONGELL, R. G. HALLIN and H. E. TOREBJÖRK, Afferent impulses in median nerve fascicles evoked by tactile stimuli of the human hand. *Brain Res.* 1970. 24 423-442.
- KUGELBERG, E., Accommodation in human nerves and its significance for the symptoms in circulatory disturbances and tetany. *Acta physiol. scand.* 1944 8 Suppl. 24.
- LEWIS, T., G. W. PICKERING and P. ROTHCHILD, Centripetal paralysis arising out of arrested bloodflow to the limb including notes on a form of tingling. *Heart* 1931 16. 1-32.
- NATHAN, P. W. Ischaemic and post-ischaemic numbness and paraesthesiae. *J. Neurol. Neurosurg. Psychiat.* 1958. 21 12-23.
- VALLBO Å. B. and K. E. HAGBARTH, Activity from skin mechanoreceptors recorded percutaneously in awake human subjects. *Exp. Neurol.* 1968 21 270-289.
- WIDDELL, G. and D. C. SINCLAIR, Pins and needles. Observations on some of the sensations aroused in a limb by the application of pressure. *J. Neurol. Neurosurg. Psychiat.* 1947 10. 26-46.
- ZOTTERMAN Y. Studies in the peripheral nervous mechanism of pain. *Acta med. scand.* 1933 80. 185-242.

epithelial lining of the crypt glands (H.-A. Hansson, pers. communication) *is* the same location as the cells of the present study.

The presence of subepithelial sensory neurons in the adult mammalian gut has not been demonstrated either, but the occurrence of subglandular small ganglion cells in the stomach of cow and pig has been described (Vau 1932). This author described the cells as small—to medium-sized, elongated, with a light, eccentric nucleus. The neuronlike cells located near the crypt epithelium in the rat small intestine appear to have similar characteristics. There is physiological evidence for a sensory nervous input to the small intestine (*cf.* Biber *et al.* 1971) and the neuronlike cells observed in this study may possibly contribute part of such sensory innervation of the gut.

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References

- BALL, A. M. and H. HYDÉN, Localization of the Thy 1 antigen in rat brain and spinal cord by immunofluorescence. *J. Neurochem.* 1978. (In press.)
- BUR, E. O. LINDQVIST and J. SUNDVALL, Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut. *Acta physiol. scand.* 1971. **82**, 177-190.
- BOHM, A. & ZACHAROWSKI, J. Sympathische Nervenzellen. *Anat. Anz.* 1896. **11**, 679-687.
- VAN, E., Über die subglandulären Ganglienzellen in der Magenwand einiger Haus- und Wildtiere. *Anat. Anz.* 1932. **71**, 340-345.



Fig. 1 Montage of electronmicrographs of a neuronlike cell (arrows) — the ileal mucosa. The cell has a light nucleus (N), two smaller processes (top) and one large process (bottom). The cytoplasm of the large process contains rough endoplasmic reticulum, mitochondria and vesicles of lysing appearance. The elongated cell is located just underneath the epithelium. Bar represents 500 nm. *Inset*: Detail from a similar neuronlike cell demonstrates multiple nexus areas between the cell and adjacent enterocytes (arrows). Bar represents 400 nm.

epithelial lining of the crypt glands (H. A. Hansson, pers. communication) *vs.* the same localization in the cells of the present study.

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References

- BRICER, A. M. and H. HYNDL, Localization of the Thy-1 antigen in rat brain and spinal cord by immunofluorescence. *J. Neurochem.* 1978. (In press.)
- LOU, B. O., LINDGREN and J. SV. VIK, Studies on the intestinal vasodilatation observed after mechanical stimulation of the mucosa of the gut. *Acta physiol. scand.* 1971. 82, 177-190.
- DEAN, A. L., Zwei Arten sympathischer Nervenzellen. *Amer. Anz.* 1896. 11, 679-687.
- YU, Z., Über die subglandulären Ganglienzellen in der Magenwand von vier Säugetiere. *Amer. Anz.* 1912. 72, 308-323.



Fig. 1 Montage of electronmicrographs of a neuronlike cell (arrows) in the ileal mucosa. The cell has a light nucleus (N), two smaller processes (top) and one large process (bottom). The cytoplasm of the large process contains rough endoplasmic reticulum, mitochondria and vesicles of stringy appearance. The elongated cell is located just underneath the epithelium. Bar represents 500 nm. *Inset:* Detail from similar neuronlike cell demonstrates multiple neuromuscular areas between the cell and an adjacent leukocyte (arrows). Bar represents 400 nm.

TABLE I. Effect of lipid peroxidation on fatty acids and phospholipids in brain cortical tissue. Paired samples of cortex homogenates were incubated with ferrous sulfate and ascorbic acid either in an aerobic (5% O₂) or in an anaerobic (100% N₂) atmosphere (for details see text). The values (mean \pm S.E.) are means \pm S.E. of 6 expts. Statistical differences were evaluated with Student's *t*-test for paired samples.

	5% O ₂	100% N ₂	Probability (p)
Total fatty acids	113 \pm 4	122 \pm 3	p < 0.01
g g ⁻¹	10.8 \pm 0.4	12.3 \pm 0.4	p < 0.01
	14.8 \pm 0.7	18.7 \pm 0.4	p < 0.001
Choline			
phosphoglyceride	22.5 \pm 0.6	26.5 \pm 0.5	p < 0.001
Choline			
phosphoglyceride	25.1 \pm 0.7	25.0 \pm 0.3	n.s.

Number of carbon atoms; number of double bonds.

Lipids were extracted with chloroform-methanol (1:1) and separated by silica gel thin-layer chromatography. Phospholipids were quantitated as P (total phosphorous) and fatty acids were analyzed by gas chromatography after conversion to methyl esters (Åkesson *et al.* 1970).

There was an appreciable amount of TBA-reactive material formed (0.638 ± 0.062 μ mol (malondialdehyde) g⁻¹ cortex) in samples incubated with oxygen, indicating peroxidation. In samples incubated with 100% N₂ the amount of TBA-reactive material was negligible (0.08 ± 0.002 μ mol (malondialdehyde) g⁻¹ cortex).

As the table shows, there were clear differences in tissue levels of phospholipids and fatty acids between the O₂-equilibrated (and peroxidized) sample and the sample tonometered with N₂. When the values were compared to zero time controls (not shown) it was apparent that phospholipid breakdown due to anaerobic phospholipase activity was undetectable. In contrast, aerobic incubation with its accompanying lipid peroxidation was followed by a decrease in choline phosphoglyceride concentration, with no change in choline phosphoglyceride, and by loss of arachidonic and docosahexaenoic acids. The latter results were similar to those previously reported to occur during lipid peroxidation in microsomal preparations from liver tissue (Recknagel and Ghoshal 1966, May and McCay 1968).

In conclusion, the present results demonstrate that selective changes in brain tissue phospholipids and fatty acids occur during peroxidation *in vitro*. It should thus be feasible to attribute the presence of lipid peroxidation *in vivo* by analyzing phospholipids and fatty acids in either the whole tissue, or in one of its subcellular fractions.

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References

- LOWRY, B. J., ELLIOTT, G., ARVIDSSON, I. Partial incorporation into rat liver glycerolipids of isotopically labeled (H) glycerol. *Biochim. Biophys. Acta* (Amst.) 1970, 270, 15-27.
- LOWRY, A. A. and F. BRENNER, Lipid peroxidation, its measurement, occurrence and significance in animal tissues. In: *Advances in Gerontological Research*, Ed. Siebeler, B. L. Academic Press, New York, 1967, 2, 335-403.

Lipid peroxidation in brain tissue *in vitro* Effects on phospholipids and fatty acids

By

EVA WESTERBERG, BJÖRN ÅKESSON, STIG REHNCRONA, DAVID S. SMITH
and BO K. SIEBÖ

It is currently debated whether or not lipid peroxidation is one of the mechanisms whereby cell damage occurs in a variety of adverse conditions, e.g. those caused by radiation, hypoxia, and carbon tetrachloride intoxication (see review by Slater 1972). When brain tissue is incubated *in vitro* in the presence of oxygen and suitable free radical initiators, appreciable amounts of lipid peroxides are formed (for literature, see Barber and Bernhei 1967). Recently it was hypothesized that the irreversible neuronal damage that occurs following incomplete cerebral ischemia is due to dislocation of free radicals and a resulting peroxidative damage to intracellular membranes (Demopoulos *et al.* 1977).

It has been difficult to prove that lipid peroxidation occurs *in vitro*. This is mainly due to the fact that the major tests used for estimating peroxidation *in vitro*, e.g. the thiobarbituric acid (TBA) method for measuring malondialdehyde and related peroxide cleavage products, is not applicable to *in vitro* situations. A promising approach is the estimation of *in vivo* changes in tissue phospholipids and fatty acids since *in vitro* studies have shown that lipid peroxidation preferentially affects ethanolamine phosphoglyceride and the polyunsaturated fatty acids, arachidonic acid and docosahexaenoic acid (Recknagel and Ghoshal 1966, Mi and McCay 1968). However, if applied to brain tissue and to changes occurring in ischemia, this approach is only valid if it can be shown that changes in fatty acids due to peroxidation are clearly different from those caused by anaerobic mechanisms. In order to test this, we incubated cerebral homogenates in the presence of free radical initiators, with and without oxygen, and determined changes in phospholipids and fatty acids.

Male Wistar rats were decapitated in liquid nitrogen. The brains were chilled out and cortical tissue (400 mg) was dissected at -22°C . The tissue was then homogenized at 0°C in 4 ml of phosphate buffer (0.05 M) containing Na⁺ in a final concentration of 0.015 M and K⁺ 0.145 M, (pH 7.0), pre-equilibrated with N₂. The homogenates were split into paired samples and tonometered at 37°C for 45 min in the presence of ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM), either with 5% O₂ or with 100% N₂. At the end of incubation, aliquots were taken for determination of TBA-reactive material (Slater and Sawyer 1971).

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Lipid peroxidation in brain tissue *in vitro* Antioxidant effects of barbiturates

By

DAVID S. SMITH, STIG REINCRÖNA, EVA WESTERBERG, BÖRÉN ÅKESSON
and BO K. SUND

Current information suggests that various barbiturates can ameliorate the adverse effects of complete or incomplete cerebral ischemia on cell viability and neurological function (for review, see Michenfelder *et al.* 1976). The mechanism(s) of protection have not been defined. Available results do not favour the view that the decrease in cerebral metabolism, which accompanies barbiturate anesthesia, can alone account for the protection (Nordstrom *et al.* 1978). It was recently suggested that free radical formation occurs during incomplete ischemia induced by middle cerebral artery occlusion in the cat, indicating that peroxidative damage to intracellular membranes is an important mechanism of cell injury. Furthermore, since methoxybenzyl inhibited the production of thiobarbituric acid (TBA) reactive material in liposomes irradiated with UV light, it was suggested that barbiturates protect brain tissue against ischemia by acting as free radical scavengers (Demopoulos *et al.* 1977, Flacon *et al.* 1978). In order to test this hypothesis we devised a system for inducing lipid peroxidation in brain tissue *in vitro* and studied the antioxidant ("scavenging") effects of several barbiturates, comparing their efficiency with that of an established free radical scavenger (promethazine).

Following decapitation of male Wistar rats, their heads were immediately frozen in liquid nitrogen, and the brains chilled out. Cortical tissue (400 mg) was dissected at -22°C and homogenized anaerobically at 0°C in 4 ml of 0.05 M phosphate buffer (pH 7.0) which had a final concentration of 0.145 M potassium and 0.015 M sodium. The brain homogenate was split into duplicate samples which were incubated for 1 h at 37°C in tonometers equilibrated with 5% O_2 in N_2 . Lipid peroxidation was enhanced by adding ferrous sulfate (0.01 mM) and ascorbic acid (0.25 mM) to the homogenates. Samples (0.5 ml) were obtained after 1, 30, 45 and 60 min of incubation. Lipid peroxidation was estimated by analyzing TBA reactive material using malondialdehyde as standard. In separate experiments, samples were obtained for analysis of phospholipid-bound fatty acids (see preceding article by Westerberg *et al.* 1979). Various compounds were tested for their ability to inhibit malondialdehyde production. These were added to one of the homogenates just prior to the addition of

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- DEMOPoulos, H., E. FLAMM, M. SELIGMAN, R. POWER, D. PIETROMORO and J. RANDOFF. Molecular pathology of lipids in CNS membranes. In *Oxygen and physiological function*. Ed. Jöbsis, F. F. Professional Information Library, Dallas, 1977. Pp. 491-508.
- MAY, H. E. and P. B. McCAY. Reduced triphosphopyridine nucleotide oxidase-catalyzed alterations membrane phospholipids. II. Enzymic properties and stoichiometry. *J. biol. Chem.* 1968. 243, 2296-23.
- SLATER, T. F. *Free radical mechanisms / tissue injury*. Plon Ltd., London, 1972.
- SLATER, T. F. and B. C. SAWYER. The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions *in vitro*. General features of the β stem used. *Biochem.* 1971. 123, 805-814.
- RECKNAGEL, R. O. and A. K. GHOSHAL. Lipoperoxidation as a vector in carbon tetrachloride hepatotoxicity. *Lab Invest* 1966. 15, 132-143.

References

- BRONKOWSKI, H. E., FLANN, M., SELIGMAN, R., POWER, D., PIETROGGIO and J. RANOWITZ. Molecular pathology of lipids in CNS membranes. In: *Oxygen and physiological function*. Ed. Jobsis, F. F. Professional Information Library Dallas, 1977. Pp. 491-508.
- FLANN, E. S., H. DEMOPoulos, M. SELIGMAN, R. G. POWER and J. RANOWITZ. Free radicals in cerebral ischemia. *Stroke* 1978, 9: 445-447.
- MCNULTZ, J. D., J. H. MILLER and T. M. SCHUB. Cerebral protection by barbiturate anesthesia. *Arch. Neurol.* 1976, 33: 345-350.
- ROSENBERG, C.-H., S. REINCHOWA, and B. K. SIESÖ. Effects of phenobarbital in cerebral ischemia. II. Alteration of cerebral energy state, as well as of glycolytic metabolites, citric acid cycle intermediates and associated amino acids after pronounced incomplete ischemia. *Stroke* 1978, 9: 335-343.
- WERNER, E., B. ÅKERST, S. REINCHOWA, D. B. SMITH and B. K. SIESÖ. Lipid peroxidation in brain tissue in vivo: effects on phospholipids and fatty acids. *Acta physiol. scand.* 1979, 105: 524-526.

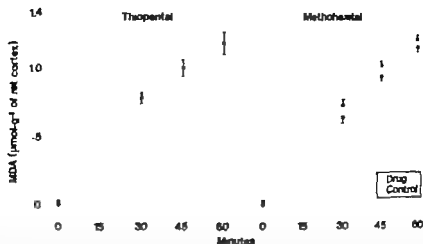


Fig. 1. Effects of thiopental or methohexital on Fe^{2+} induced lipid peroxidation in brain homogenate. Samples for analysis of malondialdehyde (MDA) were obtained after 0, 30, 45, and 60 min of incubation with $5 \mu\text{M}$ Fe^{2+} in nitrogen. The samples were paired with each brain serving as its own control. The symbols represent the mean \pm S.E. from 4 rats. Except for the zero time samples the groups are significantly different ($P < 0.05$) from their controls.

ascorbic acid and iron. Statistical analysis of the results was done using Student's *t*-test for paired samples.

When the brain homogenates were incubated without iron and ascorbic acid, moderate amounts ($0.3 \mu\text{mol g}^{-1}$) of malondialdehyde (MDA) accumulated during 1 h of incubation. In the presence of these free radical initiators, MDA production usually exceeded $1 \mu\text{mol g}^{-1}$. Thiopental (1 mM) produced 96% inhibition of MDA formation, *i.e.* the values were less than those obtained in the absence of added initiators. However, promethazine was even more efficient since it gave a comparable inhibition at a concentration of 0.1 mM. Methohexital (1 mM) produced some inhibition but its degree (8%) was much less than that produced by thiopental (Fig. 1). Phenobarbital (1 mM) or pentobarbital (1 mM) had no effect on MDA production.

Analysis of fatty acids showed that thiopental not only inhibited the production of MDA but it also inhibited the associated lipid oxidations. As described in the preceding article (Westerberg *et al.* 1979) there was an oxygen-induced loss in the sum of arachidonic and docosahexaenoic acid (18%) when the homogenates were incubated with iron and ascorbic acid. Thiopental completely prevented this change in fatty acid composition.

The present results give little support to the hypothesis that barbiturates protect the brain in ischemia by acting as free radical scavengers. Thus, whereas protection *in vivo* has been reported with thiopental, methohexital, pentobarbital and phenobarbital, only thiopental was efficient as a free radical scavenger when tested on our brain homogenate model. However, it is often difficult to extrapolate *in vitro* effects of drugs to an *in vivo* situation, and it seems necessary to study the protective effect, if any, of promethazine and related compounds before commenting further on the free radical hypothesis.

The study was supported by grants from the Swedish Medical Research Council (No. 14X 263 and 03P 4931), from the Knut and Alice Wallenberg Foundation and from the U.S. Public Health Service (No. RO1NS07838) via N.J.H. The authors are grateful to Lena Sjöberg for excellent technical assistance.

Abstracts from Meeting of the Scandinavian Physiological Society in Odense 3-4 November 1978

COMMUNICATIONS

C 1

Free Fatty Acid Mobilization Limited by Adipose Tissue Blood Flow During Exercise*

by J. BILLOW and J. MADSEN *Institut of Medical Physiology C University of Copenhagen, Denmark*

We have previously demonstrated an increase in adipose tissue blood flow (ATBF) during exercise (Billow and Madsen 1976). The purpose of the present study was to assess the free fatty acid (FFA) carrying capacity of plasma albumin under conditions where ATBF limited FFA mobilization in order to evaluate whether ATBF may be limiting for FFA mobilization during exercise.

Desclated canine subcutaneous inguinal fat was perfused at a constant rate. Lipolysis was stimulated by addition to the blood of isoprenaline 3×10^{-6} M and theophyllamine 1×10^{-4} M. In venous plasma from stimulated adipose tissue the $C_{FFA}/C_{albumin}$ ratio was 4.5 (nmol/mol) at control ATBF (3.8 ml/100 g/min). If ATBF was reduced to 70% of the control value FFA release decreased to 25%. A reduction of ATBF to 6% caused a reduction of FFA release to 10% control value. In these two situations mean $C_{FFA}/C_{albumin}$ were 6.8 and 7.4 respectively. Values >10 were never observed.

Canine and human plasma (pH 7.4) were equilibrated with heptane solutions of palmitic acid at increasing concentrations. 4-6 moles of FFA were bound to 1 mol of albumin at very low concentrations in the heptane phase. The following moles required increasingly higher concentrations in the heptane phase. No constant difference between the binding capacities of human and dog plasma was observed.

Subjects worked at a bicycle ergometer for 4 hours at 60% of their $V_{O_2 \max}$. During work their adipose tissue blood flow increased to a mean value of 10 ml/100 g/min. Average V_{CO_2} and V_{O_2} in the 4th hour were 1.59 and 1.27 l/min. Assuming a protein catabolism of 0.1 g/min, lipid metabolism can be calculated to 0.50 g/min. Taking this to be tripalmitate 1.86 mmol FFA is mobilized and metabolized per minute. Assuming 10% of the body weight to be adipose tissue, 26.6 μ mol FFA are mobilized per 100 g adipose tissue per minute. They are transported by 10-0.55 ml plasma that has a FFA concentration of 4.7 μ mol/ml before entering the tissue. With a plasma albumin concentration of 0.7 μ mol/ml, the $C_{FFA}/C_{albumin}$ in adipose tissue venous blood should be about 13-14. Thus, it appears very likely that ATBF limits FFA mobilization during prolonged heavy exercise. Calculations based on reported figures for C_{FFA} and V_{CO_2} during prolonged fasting suggest that ATBF may also be crucial for FFA mobilization in this situation.

Reference

Billow J and Madsen J *Pflügers Arch* 1976 363 231-234

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COMMUNICATIONS

C 1

Fatty Acid Mobilization Limited by Adipose Tissue Blood Flow During Exercise

B. J. BILLOW and J. MADSEN *Institute of Medical Physiology C University of Copenhagen, Denmark*

We have previously demonstrated an increase in adipose tissue blood flow (ATBF) during exercise (Billow and Madsen 1976). The purpose of the present study was to assess the free fatty acid (FFA) carrying capacity of plasma albumin, under conditions where ATBF limited FFA mobilization, in order to evaluate whether ATBF may be limiting for FFA mobilization during exercise.

Decorticated canine subcutaneous inguinal fat was perfused at a constant rate. Lipolysis was stimulated by addition to the blood of isoprenaline 3×10^{-6} M and theophyllamine 1×10^{-3} M. In venous plasma from stimulated adipose tissue the $C_{FFA}/C_{albumin}$ ratio was 15 (nmol/ml) at control ATBF (3.8 ml/100 g/min). If ATBF was reduced to 20% of the control value FFA release decreased to 25%. A reduction of ATBF to 6% caused a reduction of FFA release to 10% control value. In these two situations mean $C_{FFA}/C_{albumin}$ was 6.0 and 7.4 respectively. Values >10 were never observed.

Canine and human plasma (pH 7.4) were equilibrated with heptane solutions of palmitic acid at increasing concentrations. 4-6 moles of FFA were bound to 1 mole of albumin at very low concentrations in the heptane phase. The following moles required increasingly higher concentrations in the heptane phase. No constant difference between the binding capacities of human and dog plasma was observed.

Volunteers worked at a bicycle ergometer for 4 hours at 50% of their V_{O_2} max. During the last hour adipose tissue blood flow increased to a mean value of 10 ml/100 g/min. Average V_{O_2} and V_{CO_2} in the 4th hour were 1.59 and 1.27 l/min. Assuming a protein catabolism of 0.1 g/min, lipid metabolism can be calculated to 0.50 g/min. Taking this to be tripalmitate 1.86 μ mol FFA is mobilized and metabolized per minute. Assuming 10% of the body weight to be adipose tissue 26.6 μ mol FFA are mobilized per 100 g adipose tissue per minute. They are transported by 10×0.55 ml plasma that has a FFA concentration of 4.7 μ mol/ml before entering the tissue. With a plasma albumin concentration of 0.7 μ mol/ml the $C_{FFA}/C_{albumin}$ in adipose tissue venous blood should be about 13-14. Thus it appears very likely that ATBF limits FFA mobilization during prolonged heavy exercise. Calculations based on reported figures for C_{FFA} and V_{CO_2} during prolonged fasting suggest that ATBF may also be critical for FFA mobilization in this situation.

Reference

Billow B. J. and Madsen J. *Pflügers Arch* 1976, 363, 231-234.

Old. Part of the operating technique and data processing have been described elsewhere (Sørensen 1975, Wille 1977). The instrument ("UNIDOP") discriminates between forward and backward blood velocities by "heterodyne detection". The emitted sound beam can be either pulsed or continuous. In the pulsed mode the instrument is only sensitive to blood velocities occurring in a certain adjustable distance from the probe. Two ultrasound frequencies are available, 1.5 MHz, suitable for measurements on large deep vessels and in the heart, and 6 MHz, for measurements on superficial vessels (less than 2.5 cm from the body surface). In the present investigation 6 MHz pulsed sound was used. Velocity spectra were calculated from the doppler signal on-line in a NORD computer system. The computer also calculated the instantaneous mean velocity across the vessel lumen and the time average of this mean velocity for each successive cardiac cycle. The instantaneous mean velocity is proportional to the instantaneous blood flow through the vessel as long as certain measurement conditions are fulfilled. The proportionality constant is equal to the cross-sectional area of the vessel and thus different for different vessels. At present neither knowledge about this area or calibration against another method (e.g. plethysmograph) is needed to convert the velocity measurements to flow. However, if the relative changes in blood flow with time are interesting in itself, no calibration is necessary.

To illustrate the possible uses of this instrument we have reinvestigated the variability of the blood flow in the human hand at different environmental temperatures. This problem was originally studied by Burton (1939) with a finger plethysmograph. Our investigation confirms Burton's findings of a tremendous range of blood flow values in the fingers and of excited and rapid fluctuations in this flow in resting subjects. It does not, however, confirm Burton's description of the fluctuations as short episodes of vasoconstrictions occurring with different frequencies on basically dilated vessels.

References

- BURTON, A. C. *Am. J. Physiol.* 1939 127 437-453.
 EISEN, M., ISAKA, U. and N. SAKAMOTO. Proceedings of the III Nordic meeting on medical and biological engineering, Tampere 1975.
 LINDEN, R. P., D. W. BAKER and H. F. STROGALL. *J. Appl. Physiol.* 1966, 21 354-366.
 WILLE, S. O. *Ultrasonics*, 1977 226-230.
 SØRENSEN, J. P. *Ultrasonics in med. & biol.*, 1975 2 11-18.

C 4

Measurement of the Interendothelial Cleft Length in the Frog Mesenteric Capillary

By M. BLINDGAARD, J. FRØKJÆR-JENSEN and C. CRONE. *Institute of Medical Physiology*
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The interendothelial cleft is generally thought to be the main pathway for the transcapillary escape of small hydrophilic solutes. The total length of the cleft per unit surface area in the

C 2

Post-exercise Rise in Plasma Concentration of Free Fatty Acids (FFA) and Adipose Tissue Blood Flow

By TERJE LARSEN HARALD VIK MO KJELL MYHRE and OLE D. MØKS *Department of Physiology, Institute of Medical Biology, University of Tromsø, Norway*

Male Wistar rats were subjected to treadmill exercise for 30 minutes. Arterial concentrations of FFA, glycerol and lactate were measured before, during and after exercise. The arterial concentrations of both glycerol and lactate rose markedly during exercise and fell after its termination. The plasma concentration of FFA, however, increased only slightly during the exercise period, while a sharp increase in plasma FFA was noted upon termination of exercise; this amounted to a maximum of $1439 \pm 89 \mu\text{mol/l}$ (mean \pm S.E.M.) after 6 minutes of recovery. Thereafter plasma FFA fell. In order to study whether the post-exercise rise in plasma FFA concentration could be related to changes in adipose tissue blood flow, radioactively labelled microspheres were injected in the aorta under the various experimental conditions. This made it possible to estimate the fraction of cardiac output going to adipose tissue (FCO). FCO was reduced early in the exercise period compared with resting values, but increased up to resting values at the end of exercise. However, 3 minutes after termination of exercise, FCO was markedly increased, reaching values 2-4 times greater than the values at the end of exercise. We estimated that a simultaneous rise in adipose tissue blood flow had occurred. It is possible that during exercise, FFA concentration within adipose tissue is increased. The peak in plasma FFA concentration observed during the early recovery period might therefore be due to FFA release from adipose tissue as a consequence of increased adipose tissue blood flow in the same period.

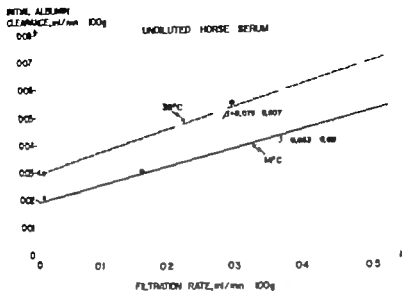
C 3

Rapid Changes in Arterial Blood Flow in Humans Measured Non-invasively by an Ultrasonic Doppler Velocimeter

By MARIANNE THORESEN and LARS WALLØE *Institute of Physiology, University of Oslo, Norway*

In the early 1960-ies Rushmer, Baker and collaborators developed an ultrasonic doppler instrument which could measure blood velocities through the skin (e.g. Rushmer et al 1966). Theoretically, the doppler signal from such an instrument should contain information about all velocities present in the vessel, and about the relative amount of blood flowing with each velocity. However, the technical problems involved have been substantial, and the instruments that are available commercially and used clinically today, operate more like "flow detectors" than as "velocity meters".

Many attempts have been made to improve the original instruments (see Woodcock 1975 for references). The instrument used in the present investigation is the result of such an attempt, and was developed in collaboration between several university departments in



0.15 ml/min \times 100 g, increasing linearly with filtration rate to some 0.07 ml/min \times 100 g at 0.5 ml/min \times 100 g of filtration. During cooling from 36°C to 14°C both CFC and initial albumin clearance \square isogravimetry decreased some 40% in due proportion to the increased viscosity of the fluid (see Figure). Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the isogravimetric capillary pressure and initial albumin clearance during isogravimetry. Initial albumin clearance at maximal vasodilatation seemed to follow the relation

$$CL = PS + \sigma(1 - \sigma) CFC \Delta TC + F(1 - \sigma)$$

where CL is the albumin clearance (ml/min \times 100 g) PS the capillary diffusion capacity for albumin (ml/min \times 100 g) F net volume flow cross the microvascular wall CFC capillary filtration coefficient (ml/min \times mmHg \times 100 g) ΔTC the colloid osmotic pressure difference across large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin. Experimentally ($1 - \sigma$ (see Figure) averaged 0.072, CFC 0.036, PS 0.009 and at a plasma colloid osmotic pressure of 70.5 mmHg CL was estimated to 0.029 during isogravimetry. It is concluded that during isogravimetry at ordinary capillary pressures the trans-vascular albumin passage is to about 70 per cent due to filtration whereas only some 30 per cent of transport takes place by diffusion both event presumably via "large pores". There was no evidence that transendothelial vascular transport should to any significant extent contribute to the passage of albumin from vessel to tissue.

mesenteric capillary is unknown. Using stereological methods to analyse electronmicroscopic pictures of transverse sections of 49 capillaries from the frog mesentery a total cleft length per μm^2 of $2400 \text{ \AA} \pm 975$ (S.D.) was found. It is possible to evaluate this figure and conclude that a large part of the interendothelial cleft must be open in order to comply with determinations of solute permeability.

Crone *et al.* (1978) using a pore model calculated an equivalent pore radius r of 110 \AA in the frog mesenteric capillary. This corresponds to an average cleft width w of 134 \AA if the passage occurs through a slit-like structure rather than through a pore ($w = r\sqrt{3}$). Knowing the cleft width the open cleft length L_o can be obtained as follows: $P = (D_o \cdot w \cdot L_o) / \Delta x$ where P_o is the potassium permeability ($67 \times 10^{-8} \text{ cm} \cdot \text{sec}^{-1}$), D_o is the free diffusion coefficient of potassium at 20°C ($1.8 \times 10^{-5} \text{ cm}^2 \cdot \text{sec}^{-1}$) and Δx the average path length in the capillary wall ($5 \times 10^{-6} \text{ cm}$). Solution for L_o gives $L_o = 1390 \text{ \AA}$ per μm^2 capillary surface area. When this figure is expressed relative to the total morphologically determined cleft length it is found that about 58% of the cleft must be open.

Lassen and Trap-Jensen (1970) from theoretical calculations concluded that in the least permeable muscle capillary 18% of the cleft was open. Perl (1971) from somewhat different assumptions arrived at a figure of 3.3% while Casley-Smith *et al.* (1975) found 4.8% for the muscle capillary.

It is likely that the junctional dimensions in various capillaries is rather constant and that variations in permeability between organs are explained by variations in the fractional open cleft lengths.

References

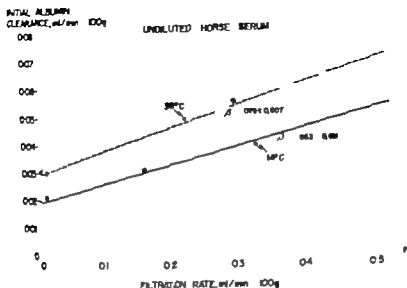
- CASLEY-SMITH J. R., H. S. GREEN, J. L. HARRIS and P. J. WADEY *Microvasc. Res.* 1975 10 43-64.
 CRONE, C. J., FROKJÆR-JENSEN, J. J., FRIEDMAN and O. CHRISTENSEN *J. Gen. Physiol.* 1976 71 195-220.
 LASSEN, N. A. and J. TRAP-JENSEN. Eds. "Capillary Permeability". C. Crone and N. A. Lassen, eds. Academic Press, New York, 1970 647-653.
 PERL, W. *Microvasc. Res.* 1971 3 223-251.

C 5

Transcapillary Passage of Albumin: Effects of Tissue Cooling and of Increases in Filtration and Plasma Colloid Osmotic Pressure

By B. RIPPE, A. KAMIYA and B. FOLKOW. Department of Physiology, University of Göteborg, Sweden.

In order to study the mechanisms behind the transfer of macromolecules across microvascular walls, initial clearance of radiolabelled serum albumin was measured in the perfused maximally vasodilated muscle vascular bed of rat hindquarters during tissue cooling, during increases in filtration and during changes in serum colloid osmotic pressure. Albumin clearance during ordinary serum perfusion at isogravimetry amounted to



0.03 ml/min \times 100 g, increasing linearly with filtration rate to some 0.07 ml/min \times 100 g at 0.5 ml/min 100 g of filtration. During cooling from 36°C to 14°C both CFC and initial albumin clearance at isogravimetry decreased some 40% in due proportion to the increased viscosity of the fluid (see Figure). Increases of the colloid osmotic pressure of the perfusate correspondingly increased both the isogravimetric capillary pressure and initial albumin clearance during isogravimetry. Initial albumin clearance at maximal vasodilatation tended to follow the relation

$$CL = PS + \sigma(1 - \sigma) CFC \Delta TC + F_v(1 - \sigma)$$

where CL is the albumin clearance (ml/min \times 100 g), PS the capillary diffusion capacity for albumin (ml/min \times 100 g), F_v net volume flow across the microvascular wall, CFC capillary filtration coefficient (ml/min \times mmHg \times 100 g), ΔTC the colloid osmotic pressure difference over large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin. Extrapolating $(1 - \sigma)$ (see Figure) averaged 0.072, CFC 0.036, PS 0.009 and at a plasma colloid osmotic pressure of 20.4 mmHg CL was estimated to 0.029 during isogravimetry.

It is concluded that during isogravimetry at ordinary capillary pressures the trans-microvascular albumin passage is to about 70 per cent due to filtration, whereas only some 30 per cent of transport takes place by diffusion, both events presumably via 'large pores'. There was no evidence that transendothelial vesicular transport should to any significant extent contribute to the passage of albumin from vessel to tissue.

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Crone *et al* (1978) using a pore model calculated an equivalent pore radius r of 110 \AA in the frog mesenteric capillary. This corresponds to an average cleft width w of 134 \AA if the passage occurs through a slit like structure rather than through a pore ($w=r/3/2$). Knowing the cleft width the open cleft length L_o can be obtained as follows $P_k=(D_k \cdot w \cdot L_o/\Delta x)$ where P_k is the potassium permeability ($67 \times 10^{-8} \text{ cm sec}^{-1}$), D_k is the free diffusion coefficient of potassium at 20°C ($1.8 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$) and Δx the average path length in the capillary wall ($5 \times 10^{-5} \text{ cm}$). Solution for L_o gives $L_o=1390 \text{ \AA}$ per μm^2 capillary surface area. When this figure is expressed relative to the total morphologically determined cleft length it is found that about 38% of the cleft must be open.

Lassen and Trap-Jensen (1970) from theoretical calculations concluded that in the less permeable muscle capillary 18% of the cleft was open. Perl (1971) from somewhat different assumptions arrived at a figure of 3.3% while Casley Smith *et al* (1975) found 4.8% for the muscle capillary.

It is likely that the junctional dimensions in various capillaries is rather constant and the variations in permeability between organs are explained by variations in the fractional open cleft lengths.

References

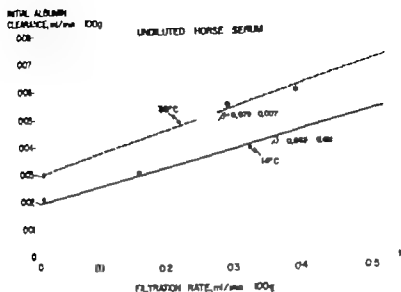
- CASLEY-SMITH J R, H S GREEN, J L HARRIS and P J WADEY *Microvasc Res* 1975 10 43-64
 CRONE C J, FROKJER JENSEN J J, FRIEDMAN and O CHRISTENSEN *J Gen Physiol* 1978 7: 195-220
 LASSEN N A and J TRAP-JENSEN In *Capillary Permeability* C Crone and N A Lassen, eds Academic Press New York 1970 647-653
 PERL W *Microvasc Res* 1971 3 223-251

C 5

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10 ml/min $\times 100$ g increasing linearly with filtration rate to some 0.07 ml/min $\times 100$ g at 0.5 ml/min $\times 100$ g of filtration. During cooling from 36°C to 14°C both CFC and initial albumin clearance at isogravimetry decreased some 40% in due proportion to the increased viscosity of the fluid (see Figure). Increases of the colloid osmotic pressure of the perfusate unexpectantly increased both the isogravimetric capillary pressure and 'initial' albumin clearance during isogravimetry. Initial albumin clearance at maximal vasodilatation seemed to follow the relation

$$CL = PS + \sigma(1 - \sigma) CFC \Delta TC + F(1 - \sigma)$$

where CL is the albumin clearance (ml/min $\times 100$ g), PS the capillary diffusion capacity for albumin (ml/min $\times 100$ g), F net volume flow across the microvascular wall, CFC capillary filtration coefficient (ml/min \times mmHg $\times 100$ g), ΔTC the colloid osmotic pressure difference across large pores (mmHg) and σ the mean osmotic reflection coefficient for albumin. Experimentally $(1 - \sigma)$ (see Figure) averaged 0.07, CFC 0.036, PS 0.009 and at a plasma colloid osmotic pressure of 70 mmHg CL was estimated to 0.029 during isogravimetry. It is concluded that during isogravimetry at ordinary capillary pressures the trans-vascular albumin passage is to about 70 per cent due to filtration, whereas only some 30 per cent of transport takes place by diffusion, both events presumably via large pores. There is no evidence that transendothelial vesicular transport should to any significant extent contribute to the passage of albumin from vessel to tissue.

Dependence of Arterial Pressure Level and of Metabolic and β Adrenergic Stimulation on Vascular Tone and Dynamic Myogenic Reactivity in the Muscle Microcirculation

By P O GRANDE and P BORGSTRÖM *Department of Physiology, University of Lund, Sweden*

Recent studies have demonstrated the existence of a rate sensitive (dynamic) component in myogenic microvascular control (Johansson and Mellander 1975, Grande *et al* 1977, 1978) which is much more effective than the previously analysed steadystate (static) myogenic response. In the present study microvascular tone and dynamic reactivity in response to a standardized increase (40 mmHg) of vascular transmural pressure (P_T) applied at a given rate ($dP_T/dt=2.5$ mmHg/s) were analysed in skeletal muscle during variation of arterial pressure (AP) and under the influence of metabolic and β -adrenergic stimuli. Graded decreases of AP, graded metabolic stimulation (muscle exercise) or graded β -adrenergic stimulation (i.e. infusion of adrenaline after α -blockade) all caused graded decreases of microvascular tone until virtual abolition at an AP level of 40 mmHg, exercise at 0.4 Hz or adrenaline infusion at a rate of $2\mu\text{g min}^{-1}\text{ kg}^{-1}$ respectively. The dynamic constrictor responses to the standardized myogenic stimulus declined in parallel with the decreasing microvascular tone during metabolic and β -adrenergic stimulation but was almost fully maintained during decreased AP. Microvascular tone at lowered AP could however not be re-established by restoration of static vascular distending pressure to normal which was experimentally produced by decreasing extravascular pressure. Microvascular tone thus is intimately depending on the prevailing AP but apparently not on its static distension effect but could rather be explained by effects of repetitive dynamic stimulation somehow induced by the AP level. Metabolic and β -adrenergic stimulation can effectively adjust microvascular tone during normal AP, most likely via their influence on dynamic myogenic reactivity and thereby can contribute to the control of capillary exchange functions. Surgical trauma and moderate haemorrhage could abolish microvascular myogenic reactivity but it was almost momentarily restored by β -adrenoceptor blockade. Stress-induced adrenaline release might thus explain the absence of myogenic reactivity and that their tone was hardly at all affected by lowered AP or by the mentioned, relatively mild metabolic and β -adrenergic stimuli.

References

- GRANDE, P-O, J LUNDVALL and S MELLANDER *Acta physiol scand* 1977 99 432-447
 GRANDE, P-O and S MELLANDER *Acta physiol scand* 1978 102 231-245
 JOHANSSON, B and S MELLANDER *Circulat Res* 1975 36 76-93

C 7

Distribution of LRH Immunoreactivity in Neural and Gastrointestinal Tissues

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Hypothalamic release and release inhibiting factors or hormones consist of a group of small polypeptides which regulate the release of anterior pituitary hormones. It has been postulated that all peptide hormone-producing cells are derivatives of the neuroectoderm and, hence, distributed in adults in the central nervous system, gastrointestinal tract and skin (Pearse 1976). Therefore, we have measured the concentrations of luteinizing hormone-releasing hormone (LRH) in various tissues of central nervous system and of gastrointestinal tract.

Outbred Sprague-Dawley rats weighing 200-300 g were used. After decapitation specimens of following tissues were removed: hypothalamus, cerebrum, cerebellum, spinal cord, pancreas, stomach, duodenum, colon and liver. The tissue specimens were weighed and homogenized in 90% methanol. After centrifugation the supernatants were evaporated and the residues were washed with 1 ml of 0.01 M acetic acid and chloroform. The upper phase was submitted to gel filtration in LH 20 (Pharmacia, Sweden, 1x40 cm column) in 100% methanol and 3 ml fractions were collected, evaporated and LRH was measured by radioimmunoassay (Seppälä, Ranta and Leppälüoto 1974). The fractions containing LRH were submitted to CM-chromatography and assayed as before.

Quantitatively highest LRH immunoreactive concentration was found in hypothalamus 250 pg per mg of wet tissue, while cerebrum had 0.6, spinal cord 0.1 and cerebellum less than 0.1 pg/mg LRH. The immunoreactive LRH concentrations of gastrointestinal tissues varied from less than 0.001 to 1 pg/mg, and was highest in the pancreas 1.0 pg/mg. In the stomach there was 0.05, duodenum 0.04 and colon 0.01 pg/mg LRH. The LRH immunoreactivity of the liver was non-detectable.

During the course of the purification the LRH immunoreactivity of the hypothalamus, cerebrum, cerebellum, spinal cord, pancreas, stomach, duodenum and colon behaved like synthetic LRH. The main function of LRH is evidently the stimulation of gonadotropin release from the pituitary, which would explain its high concentration in hypothalamus. The role of LRH in gastrointestinal tissues, if any, remains to be established.

References

- ANUZZI, A. G. E., Peptides in brain and intestine. *Nature* 1976 262 97-94.
SEPPÄLÄ, M., T. RANTA and J. LEPPÄLUOTO. In: *Biological and Clinical aspects of reproduction* (Excerpta Medica International Congress Series 4, 394 Excerpta Medica Amsterdam 1974 85-87).

Cardiorespiratory Effects of Centrally Administered TRH

By F KOIVUSALO I PAAKKARI J LEPPÄLUOTO and H KARPPANEN *Departments of Physiology and Pharmacology University of Oulu Finland*

Thyrotropin releasing hormone (TRH) is found throughout the nervous system (Leppäluoto *et al* 1978). Centrally administered TRH elevates blood pressure in the cat and rabbit (Delbarre *et al* 1977 and Beale *et al* 1977).

In this study increasing amounts of TRH (3 16 80 400 and 2000 ng per rat) or alternatively saline were injected into the lateral ventricle of urethane-anaesthetized rats. Mean blood pressure and heart rate were measured directly from the left femoral artery by means of a pressure transducer a pressure amplifier and a rate computer (Hewlett Packard). Ventilation was measured with a hot wire air flowmeter and the signals integrated with an integrator (Hewlett Packard) to obtain the tidal volume.

A dose of 3 ng TRH resulted in a significant increase in blood pressure and heart rate (Fig 1 panel A and B) whereas 16 ng was needed before ventilation rate was increased (data not shown). The increased ventilation rate paralleled a simultaneous decrease in tidal volume and hence there was no significant change in ventilatory minute volume. The cornea reflex became positive in all anaesthetized rats after 3 ng TRH but remained negative in the control rats.

Our results confirm the previously reported pressor effect of centrally administered TRH and demonstrate that TRH is one of the most potent centrally effective hypertensive agents. The lack of change in ventilatory minute volume indicates that respiratory feedback mechanisms work under influence of TRH. The physiological phenomena affected by

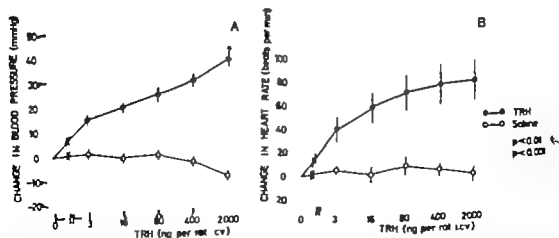


Fig 1 The effect of increasing doses of intracerebroventricularly injected TRH or saline on mean blood pressure (panel A) and heart rate (panel B). Each symbol is given as mean \pm SE (n=8) and represents the values obtained 10 min after injection. Asterisks indicate a statistical significance between TRH and the corresponding saline injections.

TRH injections are controlled by medulla oblongata and spinal cord which is in accordance with the high TRH content of these areas.

This study was supported by grants from the Orion-Yhtymä Foundation

References

- LOHMEIJER J, F. KORHONEN and R. KRAAMA, *Acta Physiol Scand* 1978 in press.
 BENJ, I. S. R. P. WHITE and SH. YU-PO HUANG, *Neuropharmacology* 1977 16 499-506.
 DOLAN, B. D. SENON and H. SCHWITT *Proc and Union Physiol Sci* 1977 13 1977

C 9

Adrenal Medullary Control of Muscular Glycogenolysis in Exercising Rats

By E. A. RITTER, H. GALBO and N. J. CHRISTENSEN *Institute of Medical Physiology B, University of Copenhagen and Department of Internal Medicine and Endocrinology, Herlev Hospital, Denmark*

We have previously shown (Galbo *et al* 1978) that in adrenomedullated rats injected with 6-hydroxydopamine (selectively destroying sympathetic nerve endings) muscular glycogen depletion during exercise is smaller than in control rats.

Now we have elucidated if this effect of sympathectomy can be ascribed selectively to the lack of either the adrenal medulla or of the peripheral sympathetic nerve endings.

Male Wistar rats weighing 180-200 g were either adrenomedullated (DM) or sham operated (C). Two weeks later unilateral extirpation of the sympathetic chain from above the second lumbar to below the second sacral ganglion was performed. During the operation skin temperature on the foot increased 1.5°C in the denervated and 0.8°C in the intact innervated leg. Seven days after sympathectomy the concentrations of norepinephrine in the sympathectomized soleus and deep vastus lateralis muscles were reduced to 9% and 11% respectively of control values.

One week after sympathectomy the rats were either forced to swim with a tail weight (2% of body weight) or were resting controls. 7 DM and 9 C-rats swam 75 min and 8 DM and 8 C-rats swam to exhaustion. Then the rats were stunned by a blow on the head and blood was collected from the carotid artery. Samples of the soleus muscles and of the liver were quickly removed.

At rest, concentrations of glucose in blood and of lactate in muscle were similar in DM and C-rats, whereas muscular and hepatic glycogen concentrations were higher in DM rats. In C-rats blood glucose had increased, muscular and hepatic glycogen had decreased. Lactate was unchanged after 5 min of swimming. At exhaustion (236 ± 10 vs. mean \pm SE) hepatic glycogen had further diminished and blood glucose had decreased below values at rest ($P < 0.05$). In DM-rats blood glucose, muscular glycogen and lactate did not change significantly during swimming. Hepatic glycogen was significantly reduced at exhaustion ($191 \text{ mmol} \pm 17 \text{ mmol}$) but was still higher than in C-rats. Sympathectomized and control legs did never differ significantly with respect to muscular glycogen and lactate concentrations.

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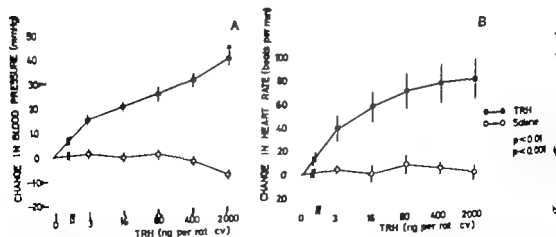


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resistance (FBF being related to muscle mass) with the result that MAP is only moderately affected. Under these conditions stroke volume usually is only affected to a minor extent (1). An increase in HR would, therefore, imply a proportional increase in CO. On this basis it might be concluded that CO is regulated according to muscle mass (and V) involved in exercise.

References

- JOSE-PETERSEN, F., L. B. ROWELL, R. G. MURRAY, C. O. BLOMQUIST, R. WITTE, E. KARLSSON, W. CORRELL and I. H. MITCHELL, *J. Appl. Physiol.* 1978, 1, press.
 ROWELL, L. B., L. HJERMANSEN and J. R. BLACKMON, *J. Appl. Physiol.* 1976, 41, 693-701.

C 11

Effects of Renal Nerve Stimulation on Arteriolar Resistances, Ultrafiltration Dynamics and Fluid Reabsorption

By HERMANSSON K., M. LARSON, Ö. KALLSKOG and M. WOLGAST, *Department of Physiology and Medical Biophysics, University of Uppsala, Sweden*

The influence of a 1 Hz and 5 Hz nerve stimulation on the hydrostatic pressure of the renal vasculature and proximal tubule, as well as the nephron filtration (SNGFR) and the nephron effective plasma flow (SNEPF) was investigated in 10 Sprague Dawley rats. During 2 Hz stimulation the plasma flow decreased from 164 ± 7.5 (mean \pm SEM) to 138 ± 8.9 nl/min and SNGFR from 54.5 ± 3.8 to 46.3 ± 7 nl/min. This was due to a 30% increase in both afferent and efferent arteriolar resistance, whereas the glomerular capillary pressure remained fairly constant at about 60 mmHg. The filtration fraction increased somewhat. The mean net driving force for filtration was 15.9 and 13.2 mmHg during these conditions, whereas the hydraulic conductivity remained unchanged at 3.4-3.5 nl/min mmHg.

The same pattern of response was seen with 5 Hz stimulation except for more marked decreases to 50% of the controls for SNEPF and SNGFR. The afferent arteriolar resistance increased 2.3 times, whereas the efferent only increased 1.5 times the controls. The fractional proximal reabsorption increased (TTP from 6.0 ± 0.39 to 7.1 ± 0.48 for control condition and 1 Hz stimulation respectively). The absolute reabsorption decreased, however, with the decreased SNGFR. It is concluded that sympathetic nerve stimulation acts in direction of fluid conservation by a decreased plasma flow and glomerular filtration and an enhanced fractional fluid reabsorption.

C 12

Arteriolar Resistance and Single Glomerular Blood Flow (SGBF) During a Maximal Tubulo-glomerular Feedback Stimulus in the Anopheles Kidney

By B. E. PERSSON and A. E. G. PERSSON, *Department of Physiology and Medical Biophysics, University of Uppsala, Sweden*

It is concluded that in exercising rats 1) adrenal medullary hormones are essential for muscular glycogen breakdown and endurance capacity 2) Sympathetic nerve activity does not significantly enhance muscular glycogenolysis 3) Exhaustion does not necessarily coincide with emptying of muscular glycogen stores

Reference

GALBO H E A RICHTER N J CHRISTENSEN and J J HOLST *Acta Physiol Scand* 1978 102 441-449

C 10

Role of Muscle Mass in the Pressor Responses to Graded Muscle Ischemia in Man

By Y SUZUKI and F BONDE PETERSEN *August Krogh Institute University of Copenhagen Denmark*

Graded muscle ischemia produced by arterial occlusion cuffs applied to the thighs during or immediately after bicycle exercise markedly influences cardiovascular function. Occlusion 30 sec before end of exercise had the most pronounced effect increasing mean arterial pressure (MAP) cardiac output (CO) and HR significantly above control values during recovery (1 %). This was attributed to a reflex mechanism elicited through the chemoreceptors in the occluded muscles. CO is diverted to skin vascular bed probably by baroreceptor activity.

In the present experiment forearm blood flow (FBF) V_o MAP HR and mechanocardiogram (MCG) (based on simultaneous tracing of ECG phonocardiogram and carotid epicutaneous pulse wave) was recorded in 3 subjects during one- or two-legged exercise in the reclined position on a bicycle ergometer. Three experimental situations were used 1) rest 7 min bicycle exercise recovery 2) rest 7 min bicycle exercise recovery with occlusion applied during the first 3 min of recovery and 3) essentially as 2) but occlusion applied 30 sec before end of exercise. These protocols were repeated twice during two-legged exercise and twice during one legged exercise.

Our data confirmed previous findings (1 %) for two-legged exercise. In addition MCG revealed that diastolic pressure time index/systolic tension time index (DPTI/TTI) decreased during both experiment 2 and 3 indicating an increased delivery/demand ratio for oxygen to the coronary circulation. This is attributed to the increase in MAP observed. Relative speed of contraction of the ventricles expressed as prejection period/ejection time (PEP/ET) did not vary.

When one- and two-legged exercise was compared similar MAP and MCG were generally observed whether or not occlusion was applied. HR was increased by 62 bpm during two-leg exercise and 34 bpm during one leg exercise and 69 and 42 bpm respectively if occlusion was applied. Occlusion however increased FBF to levels approximately twice as high during two-leg exercise as during one-leg exercise. The HR reflex appears to be muscle mass dependent while baroreceptors are evidently able to regulate peripheral

The intratubular P_{CO_2} was significantly higher than the P_{CO_2} of the arterial blood and the P_{O_2} of the efferent arteriolar blood was significantly lower than that of the arterial blood. The buffer capacity was higher and the bicarbonate concentration slightly lower for the efferent arteriolar blood than for the arterial blood.

It is concluded that a P_{CO_2} difference exists across the tubular wall and that the high intratubular P_{CO_2} favours a chemical equilibrium of the carbonic acid-bicarbonate system in the proximal tubular fluid. It is supposed that the slightly lowered bicarbonate concentration in the efferent arteriolar blood is an effect of the glomerular ultrafiltration process.

C 14

HCO_3^- transport by bullfrog duodenum

By GUNNAR FLEWSTRÖM, *Department of Physiology and Medical Biophysics, University of Uppsala, Sweden*

Recent studies have shown that gastric antral and fundic surface epithelial cells actively transport HCO_3^- into the gastric lumen. Potent inhibitors of this secretion also decrease the ability of the gastric mucosa to resist intraluminal acid. This made it of interest to examine whether the surface epithelial cells in proximal duodenum have a similar HCO_3^- transport.

The duodenal bulb and adjacent part of the duodenum was removed from bullfrogs immediately after killing of the animals by decapitation. The muscular coat was removed by blunt dissection and the mucosal tube (total length 7-8 mm) mounted between two glass tubes connected to a reservoir. The unbuffered luminal solution (5 ml) was gassed with 100% O_2 , which also gave satisfactory circulation of the solution. HCO_3^- transport was measured by continuous titration to pH 7.40 with 5 mM HCl solution under automatic control from a pH-stat equipment (Radiometer Copenhagen). A buffered solution (pH 7.38) gassed with 95% O_2 and 5% CO_2 was used on the nutrient side. The transepithelial electric potential difference was measured via two matched calomel electrodes and recorded with a high input impedance mV meter. Microscopic examination showed the absence of Brunner's glands in the present preparations.

All preparations alkalinized the luminal surface. The steady state values 60 min after starting of HCO_3^- transport and electric potential difference was $0.51 \pm 0.06 \mu eq/h$ and 18 ± 8 mV (means \pm SE, $n=12$). Elevation of nutrient calcium ion concentration from 1.8 to 7.1 mM significantly ($p < 0.01$) increased both the rate of HCO_3^- transport and the electric potential difference ($n=7$). Addition of 2,4 dinitrophenol to the nutrient side (10^{-4} M) lowered the electric potential difference and almost abolished HCO_3^- transport ($n=5$).

The results indicate that surface epithelial cells in proximal duodenum alkalinize the luminal surface. The stimulation of HCO_3^- transport by calcium ions and inhibition by 2,4 dinitrophenol is similar to that previously observed in gastric mucosa in vitro and in vivo. Surface epithelial transport of HCO_3^- may contribute to the resistance of the proximal duodenum to intraluminal acid by increasing the pH in the immediate vicinity of the luminal surface.

Earlier experiments have shown the existence of a tubulo-glomerular feedback control in the Amphiuma kidney that can reduce single glomerular filtration rate (SNGFR) at increased delivery of fluid to the distal nephron. This decrease in SNGFR could be caused by changes in afferent arterioles resistance (R_a), efferent arterioles resistance (R_e) or SGBF. Therefore these parameters were measured at zero and 50 nl/min distal perfusion rate where the high flow releases a maximal reduction of SNGFR. To calculate R_a , R_e and SGBF it was necessary to measure arterial blood pressure (AP), glomerular capillary pressure (GCP), Bowman space pressure (BSP), SNGFR, afferent and efferent colloid-osmotic pressures (π_a and π_e).

Perfusion nl/min	AP cm H ₂ O	GCP cm H ₂ O	BSP cm H ₂ O	PCP cm H ₂ O	SNGFR nl/min	π_a cm H ₂ O	π_e cm H ₂ O
0	26.0	18.7	7.0	7.5	18.0	7.7	11.9
50	25.2	14.1	4.8	7.4	7.1	7.8	11.4

At maximal feedback reduction of SNGFR from 18.0 nl/min to 7.1 nl/min SGBF was reduced from 170 nl/min to 40 nl/min. R_a did not change significantly (0.157 and 0.235 cm H₂O min/nl) while R_e increased significantly from 0.090 to 0.284 cm H₂O min/nl. Thus the SNGFR and SGBF reduction at maximal feedback response is mediated via an increased resistance in the afferent arteriole.

C 13

Pco₂ of the Superficial Structures in the Rat Kidney

By M. SOHTELL, *Department of Physiology and Medical Biophysics, Biomedica Center, University of Uppsala, Sweden*

By the ultrafiltration process in the kidney the proximal tubules are offered a load of bicarbonate. The mechanisms underlying the tubular bicarbonate reabsorption have been the subject of much discussion. One of these mechanisms generally favoured is the active hydrogen ion transport into the tubular lumen. According to this hypothesis the H⁺ and HCO₃⁻ ions react and form carbonic acid. This acid is then dehydrated forming water and carbon dioxide which are reabsorbed. The rate of this reabsorption may be influenced by various factors such as the rate of delivery of H⁺, the dehydration rate and the carbon dioxide diffusion across the tubular wall.

Recordings in vivo of the carbon dioxide tension of the proximal tubular fluid and of the efferent arteriolar blood were performed with Pco₂ microelectrodes in the rat kidney. The buffer lines of the efferent arteriolar blood and systemic arterial blood were determined with an ultramicro equilibration system and the acid-base status of the systemic arterial blood was measured.

The results support the hypothesis (Folkow et al. 1974) that genetic hypertension is associated with a narrower lumen, a thicker media and a greater contractility in the precapillary resistance vessels, but with little difference in the structure or contractile abilities of post-capillary resistance vessels.

References

- FOLKOW, B., M. HALLBACK, Y. LUNDGREN, L. WEISS, I. ALBRECHT and S. JULIUS, *A. in physiol.* 1974 90 654-656.
 JULIUS, S. M. J. and W. HALPER, *Circ. Res.* 1977 41 19-26.
 KILB, Y. M. J., P. K. HALL, SEN and C. AALKJER, *Circ. Res.* (In Press.)

C 16

Sodium Balance and Dopamine Excretion in Spontaneously Hypertensive Rats (SHR)

By H. HERLITZ, S. LUNDIN, M. HENNING, S. E. RICKSTEN, G. GÖTHBERG, M. HALLBACK and G. BERGLUND. *Departments of Physiology, Pharmacology and Med. Clin. I University of Göteborg, Sweden.*

In SHR (1) sympathetic overdrive in combination with thickening of the arterioles peripherally produces the increase in blood pressure (Lundgren 1974). In another strain of such rats the development of hypertension has been found to be associated with sodium retention (Göthberg et al. 1975). Sodium balance studies in SHR and normotensive control rats (NCR) revealed a lowered urinary sodium excretion in rats 6-7 weeks of age considered to be in the borderline phase of hypertension (Herlitz et al. 1978). There was however at this age no sodium retention since faecal sodium content was doubled.

Recently a dopamine system has been shown to be of importance in the regulation of sodium balance since this catecholamine has a pronounced natriuretic effect (Faucheux et al. 1977).

To evaluate the effect of increased sodium intake on sodium handling in SHR, NCR and Wistar Kyoto rats (WKR) a study with a higher salt intake was performed (16 mmol sodium/100 g food). Rats 6-7 weeks of age were kept in metabolic cages and fed standard diet (5 mmol sodium/100 g) for two weeks after which time the high salt diet was introduced and continued for another week. During these time periods sodium intake, urinary and faecal sodium excretion and dopamine excretion were determined (see Table I).

Urinary sodium excretion increased markedly in all groups receiving the high salt diet. This demonstrates that also the prehypertensive kidney can excrete a sodium load. There is however still a significantly lower value for SHR which could be due to enhanced renin-angiotensin activity or to increased sympathetic tone. Faecal sodium content did not increase during the high salt diet indicating that the enhanced faecal sodium excretion in SHR is not secondary to a diminished ability of the kidneys to handle salt. The increased faecal sodium excretion might be a mechanism by which the organism protects its sodium balance.

References

- FLEMSTRÖM G *Amer J Physiol* 1977 233 E1-E12.
 FLEMSTRÖM G *Acta Physiol Scand Suppl Gastric Ion Transport* 1978 111 90.
 GARNER A and G FLEMSTRÖM *Amer J Physiol* 1978 234 E535-E541

C 15

Structure and Function of Arterial and Venous Resistance Vessels In Spontaneously Hypertensive and Normotensive rats

C AALKJÆR and M J MULVANY *Biophysics Institute Aarhus University Denmark*

Segments of arterial resistance vessels (i.d. $\approx 190 \mu\text{m}$) and their corresponding veins have been taken from a precisely defined location in the mesenteric bed of normotensive (WKY) and Spontaneously hypertensive (SHR) rats (Mulvany, Hansen and Aalkjær 1978). They were mounted in vitro on a myograph capable of directly registering their wall tension while their internal circumference was controlled. All vessels were set to a normalized internal circumference L_0 close to the value for which the active wall tension was a maximum (Mulvany & Halpern 1977). At this internal circumference the media thickness m_0 was measured by light microscopy. The active wall tension ΔT_0 in response to a K-rich solution was then determined. The average results from 18 pairs of vessels are presented in Fig. 1. The active pressure Δp the vessels could have produced in vivo was calculated using Laplace's law ($\Delta p = 2\pi\Delta T_0/L_0$) and was (Mean \pm S.E.) for the arteries SHR 74.81 ± 9.85 mmHg WKY 167.59 ± 7.14 mmHg and for the veins SHR 20.33 ± 1.58 mmHg WKY 19.10 ± 2.18 mmHg.

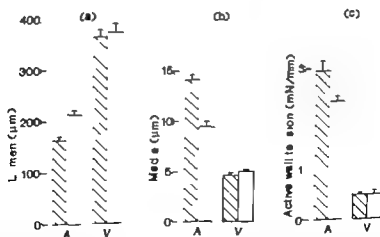


Fig. 1 (a) Normalized lumen diameter $l = L_0/\pi$ (b) media thickness at L_0 , m_0 (c) active wall tension ΔT of pairs of arterial (A) and corresponding venous (V) resistance vessels taken from 9 SHR rats (hatched columns) and 9 control WKY rats (open columns). Columns heights show means and bars show S.E. * indicates significant difference between SHR and WKY values ($P < 0.02$).

The results support the hypothesis (Folkow et al. 1974) that genetic hypertension is associated with a narrower lumen, a thicker media and a greater contractility in the precapillary resistance vessels, but with little difference in the structure or contractile ability of post-capillary resistance vessels.

References

- FELDMAN M, HALLBACK Y, LUNDGREN L, WEISS, I, ALBRECHT and S. JULIUS. *Am J Physiol* 1974 90: 654-656.
 N1. Y M J and W. HALPER. *Circ Res* 1977 41: 19-26.
 N1. Y M J, P. K. H. SHEN and C. AALKJER. *Circ Res* (In Press.)

C 16

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TABLE 1 Fractional sodium excretion in urine and faeces in NCR, SHR and WKR receiving two different diets. Urinary dopamine excretion during standard diet. Fractional urinary excretion = urinary excretion/intake $\times 100$

	Standard diet			High salt diet	
	Fractional urinary sodium excretion %	Fractional faecal sodium excretion %	Dopamine excretion ($\mu\text{g/day}$)	Fractional urinary sodium excretion %	Fractional faecal sodium excretion %
NCR (n=10)	54 \pm 2	12 \pm 1	1.3 \pm 0.1	88 \pm 2	8 \pm 1
SHR (n=10)	28 \pm 2	32 \pm	2.1 \pm 0.1	78 \pm 2	15 \pm 1
WKR (n=5)	59 \pm 4	16 \pm 3	1.1 \pm 0.1	84 \pm 2	13 \pm 1

References

- BIANCHI G, P. G. BARR, U. FOX, L. BUZZI, D. PATETTI and A. M. GIOVANETTI *Circ Res* 1973 36 Suppl. I 153-161
 FAUCHEUX B, N. T. BUU and O. KUCHEL, *Am J of Physic* 1977 232 123-127
 HERLITZ H, S. LUDIN, S. E. RICKSTEN, G. GÖTHBERG, M. AURELL, M. HALLBACK and O. BENGLUND *in press Acta Med Scand*
 LUNDGREN Y, *Acta physiol scand* 1974 Suppl. 408.

C 17

Erythrocytic Nucleoside Triphosphates Inhibit Oxygen-linked Binding of Carbon Dioxide by Carp Hemoglobin

By ROY E. WEBER *Department of Zoophysiology, University of Aarhus, Denmark*

It has long been known that in mammalian blood CO_2 is transported partly as carbamate formed by direct combination with hemoglobin. More recent studies have shown that CO binds at the π amino groups (valine residues) of the α and β chains of hemoglobin resulting in inhibitory O_2 linkage (reduction in O_2 affinity) (cf. Kilmartin and Rossi-Bernardi 1971). When protonated the α amino groups of the β chains are however also implicated in binding of anionic diphosphoglycerate (DPG) which regulates erythrocytes O_2 affinity. It is that competition for this site explains the antagonism observed by Bauer (1969) between CO_2 and DPG binding in human hemoglobin. No data appear to be available on the specific effect of CO_2 and its interaction with cofactors in fish. Although CO_2 tensions normally are low in aquatic animals many fresh water systems are hypercarbic resulting in high internal CO_2 tensions. In fishes moreover nucleoside triphosphates (NTP) substitute DPG as modulators of O_2 affinity in carps changes in both guanosine triphosphate (GTP) and ATP serve to adapt blood O_2 affinity to water O_2 tension (Weber and Lykkeboe 1978). These considerations prompted this investigation of CO_2 and NTP interactions in carp hemoglobin.

At pH values above 7.7 CO_2 distinctly depresses O_2 affinity and increases cooperativity of stripped (cofactor free) carp hemoglobin (see Fig. 1). While GTP and ATP decrease O_2 affinity this effect increases with decreasing pH resulting in increased Bohr shifts ($\Delta\log$

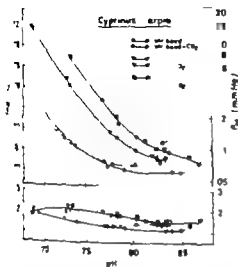


Fig. 1 Influence of CO_2 on half-saturation O_2 tension (P_{50}) and cooperativity coefficient (n) of stripped carp hemoglobin in the absence of cofactor (○ ●) and in the presence of ATP (□ ▣) and GTP (□ ▣). Open symbols and solid curves, no CO_2 ; solid symbols and broken curves, 1% CO_2 (~ 7.3 mmHg). Temperature, 20°C ; solvent Tris buffer ionic strength 0.06; hemoglobin concentration, 0.09 mM (tetramer basis); molar ratio of ATP and GTP over hemoglobin, ~ 4 .

P_{50}/pH) These data imply that as in mammals, CO_2 and cofactors bind at uncharged NH_2 and protonated $\sim\text{NH}$ groups respectively. Significantly however ATP as well as GTP completely obliterate the CO_2 effect, except at high pH (above 8.2) (Fig. 1). This suggests that carbamate will normally play an insignificant role in CO_2 transport in carp blood, and that CO_2 will have little direct influence on O_2 transport. The complete suppression of O_2 -linked CO_2 binding by cofactors contrasts to mammalian hemoglobin where DPG reduces the CO_2 effect. It implies that the β chains must be the main sites for carbamate formation in carps. This tallies neatly with the finding (Hsieh and Braunitzer 1971) that in the chains of carp hemoglobin, the N-terminal valine residue is substituted by a carboxylated serine.

References

- BLUM, C. 1969. *J. Life Sci.* 1041-1046.
 HIEH, K. and O. B. BRAUNITZER. 1971. *Hoppe-Seyler's Ztsch. physiol. Chem.* 349: 433-436.
 LIEBOWITZ, J. V. and L. ROSEN-BERNADE. 1971. *Biochem. J.* 124: 31-45.
 SCHULZ, R. E. and O. LYAKER. 1978. In press.

C 18

Glucose Transport by the Integument of a Polychaete Worm

By J. GORME, August Krogh Institute Zoophysiological Laboratory A, University of Copenhagen, Denmark.

The integument of the brackishwater polychaete *Nereis diversicolor* accumulates exogenous D-glucose from natural environmental concentrations, i.e. $< 10 \mu\text{M}$ (Ahearn & Gorme

TABLE I Fractional sodium excretion in urine and faeces in NCR, SHR and WKR receiving two different diets. Urinary dopamine excretion during standard diet. Fractional urinary excretion = urinary excretion/intake $\times 100$

	Standard diet			High salt diet	
	Fractional urinary sodium excretion %	Fractional faecal sodium excretion %	Dopamine excretion ($\mu\text{g/day}$)	Fractional urinary sodium excretion, %	Fractional faecal sodium excretion %
NCR (n=10)	54 \pm	12 \pm 1	1.3 \pm 0.1	88 \pm	8 \pm 1
SHR (n=10)	48 \pm	32 \pm	4.1 \pm 0.1	78 \pm 2	15 \pm 1
WKR (n=5)	59 \pm 4	16 \pm 3	1.0 \pm 0.1	84 \pm 2	13 \pm 1

References

- BIANCHI G, P G BARR, U FOX, L BUZZI, D PATETTI and A M GIOVANETTI *Circ Res* 1973 36 Suppl 1 153-161
 FAUCHEUX B, N T BUU and O KUCHEL, *Am J of Physic* 1977 232 123-127
 HFRITZ, H, S LUDIN, S E ROCKSTEN, G GÖTHBERG, M AURELL, M HALLBÄCK and O BENGLUND *in press Acta Med Scand*
 LUNDGREN Y *Acta physiol scand* 1974 Suppl 408.

C 17

Erythrocytic Nucleoside Triphosphates Inhibit Oxygen-linked Binding of Carbon Dioxide by Carp Hemoglobin

By ROY E. WEBER *Department of Zoophysiology, University of Aarhus, Denmark*

It has long been known that in mammalian blood CO_2 is transported partly as carbamate formed by direct combination with hemoglobin. More recent studies have shown that CO_2 binds at the α amino groups (valine residues) of the α and β chains of hemoglobin resulting in inhibitory O_2 linkage (reduction in O_2 affinity) (cf. Kilmartin and Rossi-Bernardi 1971). When protonated the α amino groups of the β chains are however also implicated in binding of anionic diphosphoglycerate (DPG) which regulates erythrocytes O_2 affinity so that competition for this site explains the antagonism observed by Bauer (1969) between CO_2 and DPG binding in human hemoglobin. No data appear to be available on the specific effect of CO_2 and its interaction with cofactors in fish. Although CO_2 tensions normally are low in aquatic animals many fresh water systems are hypercarbic resulting in high internal CO_2 tensions. In fishes moreover nucleoside triphosphates (NTP) substitute DPG as modulators of O_2 affinity in carps changes in both guanosine triphosphate (GTP) and ATP serve to adapt blood O_2 affinity to O_2 tension (Weber and Lykkeboe 1978). These considerations prompted this investigation of CO_2 and NTP interactions in carp hemoglobin.

At pH values above 7.7 CO_2 distinctly depresses O_2 affinity and increases cooperativity of stripped (cofactor free) carp hemoglobin (see Fig. 1). While GTP and ATP decrease O_2 affinity this effect increases with decreasing pH resulting in increased Bohr shifts ($\Delta \log$

partial denervation. We have repeated this experiment with electrophysiological techniques on the experimental peripheral sympathetic system.

The right superior cervical ganglion of the guinea-pig was partially denervated by cutting the preganglionic fibres that run dorsal to *arteria subclavia* (about 75%). The synaptic criterion from the remaining intact axons (running ventral to the artery in *ansa subclavia*) was measured by recording intracellularly the synaptic responses in neurones in the superior cervical ganglion while stimulating the *ansa subclavia in vitro*. In normal ganglia, the median number of fibres from this source innervating each neurone was 2.5 whereas in ganglia removed seven days after partial denervation the median increased to 3.0 ($n=8$ 6, Wilcoxon test). The median amplitude of the compound excitatory postsynaptic potential (e.p.s.p.) also increased from 6.8 mV in normal ganglia to 13.0 mV after partial denervation. Since an increase in the number of fibres innervating each neurone cannot be explained by supersensitivity or by more transmitter being released from each entry, much of the increase in the synaptic response is probably caused by sprouting of preganglionic axons with formation of new synaptic connexions.

In further experiments a stimulating electrode was placed on the cervical sympathetic trunk for 1 hr immediately after the partial denervation. To test whether the electrode caused any nerve injury the current was not turned on in a series of control animals. The results of these experiments were not significantly different from those obtained after partial denervation alone ($n=0$ 15). In the remaining animals, electrical stimulation was carried out for 1 hr at supra-maximal intensity while observing the sympathetic end organ response (5 s trains of biphasic 0.5 ms square waves ± 10 V ± 0.5 mA, 20 Hz, train rate 10 Hz). Electrical stimulation caused a substantial net increase in the synaptic responses elicited from *ansa subclavia* after seven days. The median number of fibres innervating each cell increased from 2.7 in ganglia that had been partially denervated and sham treated to 3.8 in ganglia examined after partial denervation and electrical stimulation ($n=8$ 602). The median e.p.s.p. amplitude increased from 11.0 mV to 18.1 mV.

These results show that in the superior cervical ganglion sprouting after partial denervation can be enhanced by a period of preganglionic nerve stimulation.

Reference

1. P. H. H. Axel, *J. exp. Biol. med. Sci.* 1952, 30, 341-366.

C 20

Independent Changes in the Pattern of Innervation of Focally Innervated Adult Skeletal Muscle

- B) S. P. KATTE, P. H. SANDSET and J. K. S. JENSEN, *Institute of Physiology and Institute of Anatomy, University of Oslo, Norway*

In mammals the pattern of innervation of skeletal muscle is dramatically reorganized during early postnatal development (Redfern 1970). The significance and universality of this process is not known.

1975 Gomme in prep) The aim of the present study was to characterize D-glucose transport into this tissue

By incubating groups of animals for 1-4 min in artificial sea water with 1-1000 μ l radiolabelled D-glucose influx across the apical membrane of the epidermal cells could be determined. The identity of the rate-limiting barrier was verified by suitable control experiments. The cuticle a 1-2 μ m protein polysaccharide covering on the epidermis was found to restrict diffusion of substrate to the membrane in a way comparable to but more pronounced than of an unstirred layer of medium. Diffusion of D-glucose through the intercellular epidermal spaces was negligible as compared to uptake into the cells.

The data fit the assumption of a one-component stereo-specific saturable D-glucose influx ($K \sim 100 \mu$ M $J_{max} \sim 10 \text{ mol cm}^{-2} \text{ h}^{-1}$) across the apical membrane. 100 μ M phlorizin completely blocks D-glucose uptake while phloretin is a less efficient inhibitor. D-glucose influx is Na⁺-dependent as shown by substitution of Na⁺ with choline.

Net uptake of D-glucose from low concentrations ($\sim 1 \mu$ M) determined from the rate removal of D-glucose from the incubation medium was found to be of the same magnitude as influx. Determination of net uptake was technically impossible at higher concentrations.

Under natural conditions D-glucose is rapidly phosphorylated and metabolized upon uptake into the epidermis. Sampling of blood and coelomic fluid during incubation with labelled substrate demonstrated trans-epithelial movement of D-glucose to be negligible and of the order of magnitude of trans-epithelial uptake of D-mannitol from a similar concentration.

It is suggested that D-glucose transport across the apical membrane of *Nereis* epidermis bears resemblance to that across the apical membrane of epithelia with significant trans-epithelial D-glucose movement (mammalian intestine and kidney tubule Cestoda epidermis) and thus is distinct from D-glucose uptake into non polar cells. In *Nereis* epidermis however trans-epithelial D-glucose uptake is negligible. The transport system in the apical membrane like that in the membrane of non-polar cells seems to serve only the nutrition of the transporting tissue.

Reference

AHEARN G. A. and J. GOMME, *J. exp. Biol.* 1975 62 243-264

C 19

Sprouting after Partial Denervation in the Superior Cervical Ganglion: Effect of Preganglionic Nerve Stimulation

By JAN MÄHLEN and ARILD NJÅ *Institute of Physiology University of Oslo Norway*

Cutting some of the nerve fibres innervating a target causes sprouting of the remaining intact axons. Hoffman (1952) showed histologically that in rat skeletal muscle the number of subsequent sprouts could be increased by electrical nerve stimulation at the time of

ach sound, light, vibration, smell and cold, the EC-conditions being the same as reported by Diamond et al.

The cerebral cortices of formalin-perfused brains were investigated after Klöver Barrera staining. The relative magnitude $R = \text{cortical depth/whole brain weight}$ was measured in the somesthetic and occipital areas. This magnitude can be suggested to reveal the capacity of information handling better than absolute values of cortical depth (see Jerison 1970). The results showed that 1) no significant difference in R was to be found between the IC and ICS-groups, 2) a significant decrease in R occurred in the ICS-group as compared with EC-group in part of the lateral occipital area, 3) a significant increase in R was found in the EC-group as compared with the IC-group in both lateral and medial occipital areas. The absolute values of cortical depth yielded no significant differences.

It is considered that in some of the cortical areas an optimal sensory stimulus pressure can be measured which results in an enhancement of the cortical growth. In these areas excess stimulation as well as impoverishment results in an inhibition of the cortical growth.

References

- ROBERTSON, R. M. and O. NEV-LINNA, *Internat. J. Neurosci.* 1972, 4: 171-173.
 DUNN, M. C., M. R. ROSENZWEIG, E. L. BENNETT, B. LINDER and L. LYON, *J. Neurobiol.* 1972, 3: 6-44.
 JERISON, H. J. In: *Advances in Primatology* vol. 1 (Eds. Noback, C. R. and W. Montagna). New York: Appleton-Croft Educational Division/Meredith Corporation, 1970, 225-244.

C 22

Intracellular release of Ca^{2+} May Counteract the Negative Inotropic effect of Acidosis in Myocardium

By H. GESSER and O. POUPA, Department of Zoophysiology, University of Aarhus Denmark, and Department of Clinical Physiology, University of Göteborg, Sweden

H^{+} ions allegedly inhibit the myocardial force development by competing with Ca^{2+} ions for sites on the contractile proteins (Katz and Hecht 1968). The heart of some species (e.g. flounder, turtle, snake) in contrast to other (cod, trout, carp) overcome this inhibition by some as yet unexplained compensatory mechanism (Poupa and Johansen 1975, Gesser and Poupa 1978).

A flux of Ca^{2+} from the mitochondria to the cytoplasm triggered by acidosis has been proposed as a compensatory mechanism (Gesser and Poupa 1978).

The present study on flounder myocardial strips demonstrates a compensation elicited by increased PCO_2 , but not by a decrease in HCO_3^- . Analysis of ^{45}Ca efflux rates from relaxed heart strips show nearly the double efflux rates when the myocardium is exposed to $15\% \text{ CO}_2$ compared to $3\% \text{ CO}_2$. These results are strongly suggesting that the effect of myocardial contractility during acidosis depend on an increase in the cytoplasmic Ca^{2+} activity.

We have examined the *II dig brevis I* muscle of prehatched chickens. This is a focally innervated muscle. Staining for Ach-esterase demonstrated a band of end-plates along the center of the muscle fibres. From the time of the histological appearance of end-plate esterase (13 days) there is only a single esterase site in each muscle fibre.

In 16 and 17 days old embryos more than one axon made synaptic contact with each end plate. When the nerve was stimulated at threshold intracellular recording showed unitary epp. On increasing the stimulus intensity the amplitude of the epp increased indicating recruitment of one or more additional axons innervating the same fibre. The individual components of the compound epps always had the same time course. In addition we saw smaller and slower synaptic potentials presumably due to electrical coupling between muscle fibres in the young chickens. Compound epps were also seen after stimulation of the muscle nerve at its exit from the spinal column. The threshold of activation and the conduction times of the functional motor axons varied by less than 5 percent in the 16 day old embryos. This corresponds to a distinct group of large axons in the muscle nerve.

Just before hatching at day 20 the muscle fibres were innervated only by single axons, as they are in older chickens.

We conclude that focally innervated chick muscle goes through an early developmental stage where the muscle fibres are probably polyneuronal innervation. The elimination of the redundant innervation is completed during the last week before hatching.

Reference

REDFERN P A *J Physiol* 1970 209 701-709

C 21

Reduction of the Depth of the Cerebral Cortex in the Mouse due to Excess Sensory Stimulation

By R. M. BERGSTROM, O. F. C. LINDROOS and M. L. A. RIITTINEN *Institute of Physiology, University of Helsinki, Finland*

Brain stimulation in an enriched environment, as compared with impoverished cage conditions, increases the depth of the cerebral cortex (see e.g. Diamond et al. 1972). Since it can be calculated (Bergstrom and Nevanlinna 1972) that excess activation of neural networks reduces their information handling capacity, it can be suggested that overstimulation in the young mouse would reduce the amount of cortical substance, which is responsible for the informational capacity. Therefore, experiments were made with three groups of mice (totally 62 F₁-hybrids of inbred strains C3H/BR and C57BL/6J, age 7 days) living for 39 days in three different environments: impoverished (IC), enriched (EC) and overstimulated (ICS). The specific environmental condition for the ICS-group was applied for 2 hours daily and for EC-group for 2-24 hours daily. ICS-conditions included excess stimulation

of sound, light, vibration, smell and cold, the EC-conditions being the same as reported by Cornwell et al.

The cortical cortices of formalin-perfused brains were investigated after Klöpper Barrera et al. The relative magnitude $R = \text{cortical depth/whole brain weight}$ was measured in brainstems and occipital areas. This magnitude can be suggested to reveal the capacity of duration handling better than absolute values of cortical depth (see Jerison 1970). The results showed that 1) no significant difference in R was to be found between the IC and EC-groups, 2) a significant decrease in R occurred in the ICS-group as compared with EC-group in a part of the lateral occipital area, 3) a significant increase in R was found in the EC-group as compared with the IC-group in both lateral and medial occipital areas. The absolute values of cortical depth yielded no significant differences.

It is considered that in some of the cortical areas an optimal sensory stimulus pressure can be measured which results in an enhancement of the cortical growth. In these areas overstimulation as well as impoverishment results in an inhibition of the cortical growth.

References

- Jerison, R. M. and O. NEVAILINDA, *Isavm. J. Neurosci.* 1972, 4: 171-173
 Perna, M. C., M. R. ROSZKOWICZ, E. L. BENDRETT, B. L. ODER and L. LYON, *J. Neurobiol.* 1972, 3: 7-14
 Jerison, R. J. in *Advances in Primatology* vol. 1 (Eds. Noback, C. R. and W. Montagna) New York: Appleton-Century-Crofts Educational Division/Meredith Corporation, 1970, 225-244

C 22

Intracellular release of Ca^{2+} May Counteract the Negative Inotropic effect of Acidosis in Ruminants

by H. GESSER and O. POUPA, Department of Zoophysiology, University of Aarhus, Denmark, and Department of Clinical Physiology, University of Göteborg, Sweden

Fluoride allegedly inhibit the myocardial force development by competing with Ca^{2+} ions for the contractile proteins (Katz and Hecht, 1968). The heart of some species (e.g. turtle, turtle snake) contrast to other (cod, trout, carp) overcome this inhibition by an, as yet unexplained, compensatory mechanism (Poupa and Johansen, 1975; Gesser and Poupa, 1978).

Efflux of Ca^{2+} from the mitochondria to the cytoplasm triggered by acidosis has been suggested as a compensatory mechanism (Gesser and Poupa, 1978).

The present study on flounder myocardial strips demonstrates compensation elicited by increased PCO_2 but not by a decrease in HCO_3^- . Analysis of ^{45}Ca efflux rates from isolated heart strips show nearly the double efflux rates when the myocardium is exposed to 15% CO_2 compared to 3% CO_2 . These results are strongly suggesting that during myocardial contractility during acidosis depend on an increase in the cytoplasmic Ca^{2+} activity.

References

- GESSER H and O POUPA The role of Intracellular Ca under hypercapnic acidosis of cardiac muscle Comparative aspects *J comp Physiol* (in press)
- KATZ A M and H H HECHT Editorial *Am J Med* 1969 47 497-502.
- POUPA O and K JOHANSEN *Am J Physiol* 1975 228 684-688.

C 23

Cardiovascular Control Mechanisms During Isometric Contractions— a One-leg Strength Training Model

By ELSE NYGAARD STEVEN LEWIS JORGE SANCHEZ and BENGT SALTIN *Arhus Krogh Institute University of Copenhagen Denmark*

The classical concept has been that the blood pressure (BP) and heart rate (HR) responses to isometric contraction are dependent on the relative force (percentage of maximum voluntary contraction force MVC) exerted by the contracting muscles (Lind and McNicol 1967). However, there is recent evidence that the responses elicited may also in some cases be related to the absolute force exerted (Mitchell et al 1977).

The present study was designed to determine whether absolute or relative force exerted is of primary importance and to examine the interplay of central versus peripheral control mechanisms for the BP and HR responses. This was accomplished by testing the knee extensor muscles of each leg of 9 healthy men before and after the muscles of one leg had been strength trained. The subjects performed 120 maximal isometric knee extension contractions/day lasting 5 sec each 4 days/week for 9 weeks. Direct BP (brachial artery) HR and rectified electromyography (EMG) of both legs were recorded continuously during 2 min isometric knee extension contractions with each leg.

MVC before and after training was 56 ± 11 and 68 ± 9 kg ($p < 0.05$) in the trained leg (TL) and 56 ± 6 and 59 ± 8 kg in the untrained leg (UTL) respectively. No differences in BP HR and EMG were observed during contraction with TL or UTL when each was tested at the same relative load (40% MVC) before and after training. After training mean BP HR and EMG were lower than before training with TL and UTL when each was tested at the same absolute load. And when TL was compared with UTL after training at the same absolute load all above variables were lower with TL (see Table which gives mean \pm SD at man 2 test $+ = p < 0.05$).

		TL		UTL		TL vs. UTL
Mean BP (mmHg)	pre	152 (23)	+	152 (16)	+	TL 133 (5)
	post	131 (9)		133 (7)		UTL 143 (10)
HR (beats/min)	pre	108 (16)	+	117 (19)	+	TL 97 (12) +
	post	97 (13)		106 (18)		UTL 108 (18)
EMG (arb. units)	pre	1.6 (0.2)		1.8 (0.5)		TL 1.4 (0.3) +
	post	1.3 (1.2)		1.6 (0.4)		UTL 1.9 (0.3)

The present results clearly demonstrate that BP and HR responses to isometric contraction with a given muscle group are primarily dependent on the relative rather than the absolute force exerted by the contracting muscles. The pronounced reductions in BP HR

and EMG during contraction with both TL and UTL at the same absolute load after training suggests that strength training reduces the influence from the motor cortex (central command). However, the lower BP, HR and EMG responses of TL as compared with UTL at the same absolute load also point to peripheral factors influencing cardiovascular regulation.

One possible interpretation of the present findings is that although central command may be lower with both TL and UTL after training there is an additional reduction in central command with TL. The hypertrophy of trained muscle and the increased maximal force development in our subjects (muscle biopsy and computerized tomography scanning data) may require fewer motor units to be activated at the same absolute load in TL.

References

- LEA, A. R. and G. W. McNICOLL. *J. Physiol.* 1967 192: 595-607.
 McNICOLL, J. H. B., SALTIN, F. C., PAYNE, B., SCHUBERT, T. L., HANSEN, and J. H. CHRISTENSEN.
Proceedings of the 1 International Union of Physiological Sciences, Paris, 1977

C 24

Discrimination of Enantiomers in Olfaction

By H. MUSTAPARTA, M. E. ANGST and G. H. LANIER. *Institute of Biology, Odense University, Denmark*

Discrimination of enantiomers in olfaction is of interest because of their identical chemical properties, but steric differences. The present study deals with receptor discrimination of pheromone enantiomers which cause dramatical differences in the behavior of bark beetles. It thus have played a decisive role in the evolution of the reception. One species *Ips paraconfusus* Lanier produce the (+)-ipsdienol(2-methyl-6-methylene 2,7-octan-4-ol) which is important for the attraction while the (-)-form has no effect (Vité pers. commun). Another species *I. posticus* shows variations of two geographical isolated populations. The western (USA) population living together with *I. paraconfusus* produce the (-)-form, while the eastern one produce a mixture of the two isomers. The attractive effect of the two enantiomers seems to be in accordance with the production (Lanier in progress). The present study was made to disclose the underlying receptor discrimination which accounts for these behavioral effects.

We have recorded responses from single olfactory cells in these species while stimulating with both enantiomers and the racemic mixture. All cells activated by one form, were also activated by the other. By using a syringe olfactometer and double series of each stimulant significant differences of the stimulation effect appeared. One enantiomer was commonly 10 times more effective than the other. Thus, in *I. posticus* most of the ipsdienol cells were more sensitive to the (-)-form than to the (+)-form, while the opposite appeared to be the case for *I. paraconfusus*. The racemic mixture showed an intermediate effect. These

differences in receptor sensitivities may explain the behavioral differences of *I. paraconfusus* and the western *I. pini*. The behaviour of the eastern *I. pini* is more complicated to explain. It seems that the other "lipoic acid cells" play a role in the central pathways resulting in their preferences for the (+) form or the racemic mixture. In general the discrimination of these enantiomers seems to be due to both an interaction on the receptors as well as an interaction in the central pathways.

C 25

Role of C3-C4 Proprioceptive Neurons in Forelimb Movements in the Cat

By H. ALSTERMARK, A. LUNDBERG, U. NORRSELL and E. SYBIRSKA, Department of Physiology, University of Göteborg, Sweden

Higher centres excite forelimb motoneurons disynaptically via proprioceptive neurons (PN) which take origin in the C3-C4 segments. These PN's are characterized by monosynaptic convergence mainly from descending pathways, e.g. corticospinal, rubrospinal, tectospinal and bulbo-spinal tracts, but also from forelimb afferents (Illert et al. 1977, 1978). Disynaptic inhibition evoked from the same sources may serve as feed forward inhibition giving spatial selectivity in activation of the PN's (Illert et al. 1975). The C3-C4 PN's presumably form a premotoneuronal spinal centre which integrates information from many sources. We have attempted to elucidate their role by investigating movements in cats after spinal cord lesions.

Motor proficiency was tested by means of the ability to take food from the bottom of a cylinder (height 40 mm, diameter 30 mm) placed vertically on the floor or horizontally 150 mm above the floor level. Using these tests, Gorska and Sybirska (in press) found severe impairment of contralateral forelimb movements after transection of the pyramid and destruction of the red nucleus.

The dorsal or ventral part of the lateral funiculus was transected in C2 or C5. The dorsal C2 lesion was made in order to interrupt rubrospinal and corticospinal connexions both to the PN's and the forelimb segments. The dorsal C5 lesion interrupts the connexions to the forelimb segments without interfering with the input to the C3-C4 PN's or their ventrally located axonal projections to forelimb motoneurons (Illert et al. 1978). The dorsal C2 lesion gave severe motor defects similar to those found after lesions of the pyramid and the red nucleus (Gorska and Sybirska in press). In contrast, animals with dorsal C5 lesions placed the forepaw rapidly and accurately into the cylinder already 10 days after surgery. Movements of digits were deficient during the entire postsurgical periods (3 months).

After a ventral C5 transection the cats lifted the forelimb in the direction of the cylinder but there was marked ataxia. Placing the forepaw in the cylinder often required several adjusting movements. Once having reached the cylinder the animals performed the movements of the digits and the forepaw supination required to take the food. No defects of forelimb movements were observed after a ventral C2 lesion which interrupted other fibre systems with the same transverse location as the proprioceptive axons.

The results suggest that the C3-C4 propriospinal system is utilized in precise rapid movements of the forelimb. Fine motor control of digits seems to operate through interneuronal systems located within the forelimb segments.

References

- LIETZ, M. A., LINDBERG, Y. and R. TANAKA, *Exp. Brain Res.* 1977, 29, 323-346.
 LIETZ, M. A., LINDBERG, Y., PADEL, R. and R. TANAKA, *Exp. Brain Res.* 1978, In press.
 LIETZ, M. A., LINDBERG, Y., PADEL, R. and R. TANAKA, *Brain Res.* 1975, 93, 530-534.
 LIETZ, M. A., LINDBERG, Y. and R. TANAKA, *Exp. Brain Res.* 1978, In press.
 GROSS, T. and E. SYRBECKA, *Acta Neurobiol. Exp.* In press.

C 26

Effect of Amiloride on the Mechanosensitivity of Hair Cells in the Lateral Line System of *Xenopus laevis*

By FINN JØRGENSEN, *Institute of Physiology, Odense University, Denmark*

In the model for the transducer mechanism of hair cells Davis (1965) suggested that a leakage current across the transducer membrane was modulated by changes in membrane conductance and that these were governed by movements of the sensory hair of the cells.



Fig. 1. A schematic drawing of a patch showing two of the neuromasts each represented by two hair cells of opposite polarity. Action potentials were recorded with glass microelectrodes close to the basal neuromast and neuromasts more caudally were mechanically stimulated by moving glassball attached to loudspeaker. B. The bottom traces show the driving voltage to the loudspeaker (20 Hz). The voltage also triggered the sweep of the averaging computer. The upper traces show the probability of occurrence of afferent spikes related to the phase of the mechanical stimulation obtained in Ringer solution and in Ringer + 9×10^{-6} M Amiloride. The duration of the square pulse triggered by the spikes and trigger 50 times, in 5 msec. The middle traces show the averaged spikes obtained in the 1st solution. The amplitude of the spikes did not change during the experiments. Calibration: 5 mV and 10 msec.

Ca and K ions have been suggested as charge carriers across the transducer membrane (Sand 1975 Russel and Sellick 1976). A leakage current across the transducer membrane has not been demonstrated but might depend upon an energy dependent transport of NaCl across the sensory epithellium.

The effect of Amiloride on the mechanosensitivity of the free standing hair cells in the lateral line system of *Necturus mac* has been investigated. Fig 1B shows an example of this effect on the probability for occurrence of afferent spikes from the stich relative to the phase of optimal mechanical stimulation (POS). The change in height of POS reflects the effect of Amiloride. In 31 experiments the average change in POS was $45\% \pm 4\%$ (SEM). Substitution of Na by choline or removal of NaCl did not significantly affect the mechanosensitivity. In solutions of low or zero NaCl 9×10^{-6} M Amiloride reduced the POS by $38\% \pm 5\%$ (SEM) $n=79$. The effect of Amiloride was reversible and at concentrations of 1.2×10^{-6} M the afferent spikes were not phase locked to the mechanical stimulation.

The effect of Amiloride on the mechanosensitivity might suggest that the permeability of the outward facing membrane of the hair cell to Na is of importance to the ionic mechanism of the hair cells. The lack of effect by the removal of Na from the solution however questions the specificity of Amiloride in this system in contrast to findings in frog skin.

References

- DAVIS H. *Cold Spr Harb Symp quant Biol* 1965 30 181-90
 RUSSEL J J and P M SELICK *J Physiol* 1976 257 45-55
 SAND O *J comp Physiol* 1975 102 27-42.

C 27

Transfer Properties of the Slowly Adapting Stretch Receptor of the Crayfish Abdomen

By GUNNAR SYDNES *Institute of physiology University of Oslo Norway*

I have studied the transfer properties of the slowly adapting stretch receptor of the crayfish abdomen (see Alexandrowicz 1967) by introducing small sinusoidal variations in the length of the receptor muscle. There was reason to believe that the isolation procedure used by previous investigators (Borsellino et al 1965 Brown & Stein 1966) might interfere with the properties of the receptor (see Njå & Walløe 1973). Therefore the receptor was left in situ. Reflex input to the receptor was eliminated by cutting its central connection and the cut end of the nerve was drawn into a suction electrode for registration of receptor activity.

The sinusoidal variation on the input was generated by a feed-backcontrolled electromagnetic stretcher. The movements of that stretcher was transferred to a glass rod which was fastened to the 4 tergite of the isolated abdomen thus stretching the receptor of the third abdominal segment. In most experiments the sinusoidal variation was superimposed on a steady flexion in the joint of about 30° . This regularly resulted in a

mean firing-rate of 10–15 imp/sec. The preparation was bathed in Ringer solution with temperature about 11°C

The position in the joint investigated was measured by means of two position transducers. Signals from these together with the nerve signals were fed into a NORD-1 computer using a procedure finding the 0 and 1 harmonic analytically both the mean and the 1 harmonic of both input and output could be rapidly calculated

I first investigated the linearity of the receptor by applying signals with different amplitudes to the input of the receptor. The amplification of the system proved to be surprisingly constant over a large range of input amplitudes. Accordingly the system should be well suited for transfer property investigations

The transfer properties of the receptor organ were investigated by stimulating the receptor with low-amplitude signals with different modulation frequencies. Small amplitudes are used to prevent phase locking of the receptor response. It was then possible to measure the amplitude of the impulse train even when the modulation frequency was well above the mean firing-rate of the stretch receptor

The amplification of the system as function of modulation frequency showed a regular behaviour from one experiment to another. There was a characteristic maximum in amplification at about 5 Hz modulation frequency the maximum being about 3 times the amplification found for very low modulation frequencies. When the modulation frequency was increased above 5 Hz, the amplification always fell rapidly towards zero

Within the limits of linearity the receptor of our preparation shows a characteristic behaviour also found by previous investigators on isolated receptors (Borsellino et al. 1964). However our results suggest that more stable and reproducible results can be obtained by leaving the receptor *in situ* than has previously been found in isolated preparations

References

- LEI WEINSTEIN, J. S. *Biol Rev.* 1967, 42, 229–236
 BORSELLINO, A., POPPEL, R. E. and TERLOLO, C. A. *Cold Spring Harbor Symp Quant Biol* 1965, 30, 561–566
 LEI, M. C. and R. B. STEIN. *Kybernetik* 1966, 3, 175–185
 LEI, A. and L. WAI. *Rev. J. Neurophysiol* 1973, 26, 106–1070

C 28

Effect of Membrane Polarization in Motoneurons on Timing of Antidromic Invasion

By J. LIPMAN, Department of Physiology, University of Göteborg, Sweden

Changes in latency of antidromic action potentials recorded extracellularly from single motoneurons have been used as an indirect measure of neuronal excitability (Merrill 1974, Lipki et al. 1977, 1978). This approach was based on the observation that the delay between the initial segment (IS) and soma-dendritic (SD) spikes recorded intracellularly from motoneurons is shortened by depolarization and lengthened by hyperpolarization of the cell body (Brook et al. 1953).

The latency to the extracellularly recorded antidromic action potentials might be used to estimate the level of membrane polarization in a more quantitative way provided that the relation between the IS-SD transition time and some polarization were known in greater detail. It should also be important to know whether the IS-SD delay were solely responsible for the variation in the latency of the extracellularly recorded antidromic spikes.

To analyse the timing of antidromic invasion delays between stimuli applied to the ventral root and the onsets of the IS and SD spikes were measured while recording intracellularly from lumbar motoneurons. Depolarizing and hyperpolarizing current pulses (15–30 ms, 1–20 nA) were applied to displace the membrane potential; the membrane potential and the input resistance were calculated using the spike height method (Frank and Fuortes 1956). In all motoneurons studied there was approximately linear relationship between the IS-SD delay and soma polarization for both depolarizing and hyperpolarizing directions. However, latency increase during hyperpolarization was larger ($0.40 \text{ ms}/10 \text{ mV} \pm 0.18$, $n=12$) than the rate of latency decrease during depolarization ($0.16 \text{ ms}/10 \text{ mV} \pm 0.12$, $n=14$). The larger changes of the IS-SD delay in hyperpolarizing direction could be explained in part by the conductance increase occurring with hyperpolarization (anomalous rectification). The delay from the stimulus to the IS spike changed in the same direction as the IS-SD delay but the rate of changes was smaller (mean $0.17 \text{ ms}/10 \text{ mV}$ for hyperpolarizing and $0.05 \text{ ms}/10 \text{ mV}$ for depolarizing currents). This suggests that the antidromic invasion of the IS region and/or conduction velocity in the proximal part of the axon also may be influenced by membrane potential in motoneurone soma.

The shifts in latency of SD spikes in motoneurons evoked by polarizing current pulses are comparable to the fluctuations of latency of antidromic invasion observed in brainstem neurons whose membrane potential was modified by synaptic inputs (cf. Merrill 1974; Lipski et al. 1977, 1978). The present results suggest that recording of antidromic latency changes may be a particularly sensitive method for detecting inhibition of neurones in extracellular studies.

References

- BROCK, L. G., J. S. COOMBS and J. C. ECCLES. *J. Physiol.* 1953, 122, 429–461.
FRANK, R. and M. G. F. FUORTES. *J. Physiol.* 1956, 134, 431–470.
LIPSKI, J., J. H. COOTE and A. TRZESKI. *Brain Res.* 1977, 135, 162–166.
LIPSKI, J., A. TRZESKI and L. KUBIN. *Brain Res.* 1978, in press.
MERRILL, G. In: *Essays on the Nervous System*. Clarendon Press, Oxford, 1974, pp. 451–486.

C 29

Differentiated Adrenergic Responses of Human Oviductal Muscle Layers to Noradrenaline and Transmural Nerve Stimulation

By B. LINDBLOM, B. LJUNG and L. HAMBERGER. *Departments of Physiology and Obstetrics and Gynecology, University of Göteborg, Sweden*

Studies on ovum transport in the human oviduct have demonstrated a fast passage through the distal segments followed by a delay for 2–3 days at the proximal end of the ampullary

portion (Cronatio *et al.* 1977). A sphincter-like function may therefore be attributed to the cervical-isthmic junction (AIJ) which has a rich supply of adrenergic nerves (Owman *et al.* 1967). *In vitro* studies on small strips from the two major smooth muscle layers of the human AIJ (Liedblom *et al.* 1978) revealed that adrenaline as well as noradrenaline (NA) cause inhibition of the spontaneous activity in strips from the inner circular layer but increase contractile activity in strips from the external, longitudinal layer. The present investigation was initiated to compare the effects of NA with those of transmural nerve stimulation (TNS) on this *in vitro* system.

Human oviducts were obtained at operation and millimeter wide muscle strips were prepared from both the circular and longitudinal layers. The preparations were mounted in an organ bath and contractile activity was recorded isometrically. Atropin was added to the medium to avoid possible influence of cholinergic nerves.

TNS (6 Hz, 15 V, 4 ms) caused relaxation of strips from the circular layer but increased activity of longitudinal strips. Responses of similar character and magnitude were elicited by addition of NA ($1.0 \mu\text{M}$). β -adrenoceptor blockade by propranolol abolished the inhibitory response of the circular muscle to NA and augmented the stimulatory effects of NA and TNS in the longitudinal strips. The inhibitory response of the circular strips to TNS was not constantly blocked by propranolol, which seems to indicate a non-adrenergic inhibitory neurotransmitter. The α -adrenoceptor antagonist phenoxybenzamine blocked the stimulatory response of the longitudinal specimens to both TNS and NA. Pretreatment with tetrodotoxin abolished the effects of TNS but not the response to NA.

The present results give additional evidence for a differentiated distribution of α - and β -adrenoceptors in the smooth muscle layers of the human oviductal isthmus. The longitudinal layer exhibits marked dominance of α -receptors whereas in the circular layer the β -receptors are predominant. If this concept is applicable in the *in vivo* situation a properly timed adrenergic activation might play a facilitating role for the passage of the fetus through the AIJ by an increased contractility of the longitudinal muscle layer in combination with a relaxation of the internal circular layer. Furthermore such relaxation may be obtained by a non-adrenergic neurogenic mechanism.

References

- CROATIĆ, H. B., M. E. ORTIZ, M. SOLEDAD DIAZ, R. HESS, J. P. BALMACEA, S. CHEVIAKOFF and C. LUND, *Fertil. Steril.* 1977 28 283.
LUND, B., L. H. VONZIGER and B. LUND, *Acta physiol. scand.* 1978 102 9A.
OWMAN, C. E., R. ROSENBERG and N. O. SJÖBERG, *J. Ob. Gyn.* 1967 30 763.

C 30

Adrenergic and Cholinergic Responses to Transmural Nerve Stimulation in Rat Uterus *in Vivo*

By S. E. DAHLÉN and P. HEDQVIST From the Department of Physiology Karolinska Institute Stockholm S-141 86

The latency to the extracellularly recorded antidromic action potentials might be used to estimate the level of membrane polarization in a more quantitative way provided that the relation between the IS-SD transition time and some polarization were known in greater detail. It should also be important to know whether the IS-SD delay were solely responsible for the variation in the latency of the extracellularly recorded antidromic spikes.

To analyse the timing of antidromic invasion delays between stimuli applied to the ventral root and the onsets of the IS and SD spikes were measured while recording intracellularly from lumbar motoneurons. Depolarizing and hyperpolarizing current pulses (15–30 ms 1–20 nA) were applied to displace the membrane potential. The membrane potential and the input resistance were calculated using the spike height method (Frank and Fuortes 1956). In all motoneurons studied there was approximately linear relationship between the IS-SD delay and soma polarization for both depolarizing and hyperpolarizing directions. However, latency increase during hyperpolarization was larger ($0.40 \text{ ms}/10 \text{ mV} \pm 0.18$, $n=12$) than the rate of latency decrease during depolarization ($0.16 \text{ ms}/10 \text{ mV} \pm 0.12$, $n=14$). The larger changes of the IS-SD delay in hyperpolarizing direction could be explained in part by the conductance increase occurring with hyperpolarization (anomalous rectification). The delay from the stimulus to the IS spike changed in the same direction as the IS-SD delay but the rate of changes was smaller (mean $0.12 \text{ ms}/10 \text{ mV}$ for hyperpolarizing and $0.05 \text{ ms}/10 \text{ mV}$ for depolarizing currents). This suggests that the antidromic invasion of the IS region and/or conduction velocity in the proximal part of the axon also may be influenced by membrane potential in motoneurone soma.

The shifts in latency of SD spikes in motoneurons evoked by polarizing current pulses are comparable to the fluctuations of latency of antidromic invasion observed in brainstem neurons whose membrane potential was modified by synaptic inputs (cf. Merrill 1974; Lipski et al. 1977, 1978). The present results suggest that recording of antidromic latency changes may be a particularly sensitive method for detecting inhibition of neurons in extracellular studies.

References

- BROCK, L. G., J. S. COOMBS and J. C. ECCLES, *J. Physiol.* 1953, 122, 429–461.
FRANK, K. and M. G. F. FUORTES, *J. Physiol.* 1956, 134, 451–470.
LIPSKI, J., J. H. COOTE and A. TRZĘBSKI, *Brain Res.* 1977, 135, 162–166.
LIPSKI, J., A. TRZĘBSKI and L. KUBIN, *Brain Res.* 1978, in press.
MERRILL, G. Ed., *Essays on the Nervous System*, Clarendon Press, Oxford, 1974, pp. 451–486.

C 29

Differentiated Adrenergic Responses of Human Oviductal Muscle Layers to Noradrenaline and Transmural Nerve Stimulation

By B. LINDBLOM, B. LJUNG and L. HAMBERGER, Departments of Physiology and Obstetrics and Gynecology, University of Göteborg, Sweden

Studies on ovum transport in the human oviduct have demonstrated a fast passage through the distal segments followed by a delay for 2–3 days at the proximal end of the ampullary

junction (Crouzet *et al.* 1977). A sphincter-like function may therefore be attributed to the myotary-isthmic junction (AIJ), which has a rich supply of adrenergic nerves (Owman *et al.* 1967). *In vitro* studies on small strips from the two major smooth muscle layers of the human AIJ (Lindblom *et al.* 1978) revealed that adrenaline as well as noradrenaline (NA) cause inhibition of the spontaneous activity in strips from the inner circular layer but increase contractile activity in strips from the external longitudinal layer. The present investigation was initiated to compare the effects of NA with those of transneuronal nerve stimulation (TNS) on this *in vitro* system.

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TNS (6 Hz, 15 V, 4 ms) caused relaxation of strips from the circular layer but increased activity of longitudinal strips. Responses of similar character and magnitude were elicited by addition of NA ($1.0 \mu M$). β -adrenoceptor blockade by propranolol abolished the inhibitory response of the circular muscle to NA and augmented the stimulatory effects of NA and TNS in the longitudinal strips. The inhibitory response of the circular strips to TNS was not constantly blocked by propranolol, which seems to indicate a non-adrenergic inhibitory neurotransmitter. The α -adrenoceptor antagonist phenylephrine blocked the stimulatory response of the longitudinal specimens to both TNS and NA. Pretreatment with tetrodotoxin abolished the effects of TNS but not the response to NA.

The present results give additional evidence for a differentiated distribution of α - and β -adrenoceptors in the smooth muscle layers of the human oviductal isthmus. The longitudinal layer exhibits a marked dominance of α -receptors, whereas in the circular layer the β -receptors are predominant. If this concept is applicable in the *in vivo* situation, a properly timed adrenergic activation might play a facilitating role for the passage of the ovum through the AIJ by an increased contractility of the longitudinal muscle layer in combination with a relaxation of the internal circular layer. Furthermore, such relaxation may be obtained by a non-adrenergic neurogenic mechanism.

References

- CROUZET, H. B., M. E. ORTIZ, M. SOLEDAD DIAZ, R. HESS, J. P. BALMACEA, S. CHEVIAKOFF and C. LLADO, *Fertil Steril* 1977 28 283.
LINDBLOM, B., L. HANSSON and B. LJUNG, *Acta physiol. scand.* 1978 102 9 A.
OWMAN, C. E., R. RONDORF and N. O. SJÖBERG, *J. Ob. Gyn.* 1967 30 763.

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References

- BROCK, L. G., J. S. COOMBS and J. C. ECCLES. *J. Physiol.* 1953, 122, 479–461.
FRANK, H. and M. G. P. FUORTES. *J. Physiol.* 1956, 134, 451–470.
LIPSKI, J., J. H. COOTE and A. TRZESKI. *Brain Res.* 1977, 135, 16, 166.
LIPSKI, J., A. TRZESKI and L. KUBIN. *Brain Res.* 1978, In press.
MERRILL, G. In *Essays on the Nervous System*, Clarendon Press, Oxford, 1974, pp. 451–486.

C 29

Differentiated Adrenergic Responses of Human Oviductal Muscle Layers to Noradrenaline and Transmural Nerve Stimulation

By B. LINDBLÖM, B. LJUNG and L. HAMBERGER. *Departments of Physiology and Obstetrics and Gynecology, University of Göteborg, Sweden*

Studies on ovum transport in the human oviduct have demonstrated a fast passage through the distal segments followed by a delay for 2–3 days at the proximal end of the ampullary

(Wolansson, Glise and Glise 1978) nociceptive stimuli in the gastroduodenal area was found to suppress gastric motility by activation of vagal non-adrenergic inhibitory fibres to the stomach. The present study was designed to explore the possible reflex pathway for vagal gastric inhibition in connection with nociceptive stimuli in the small intestine.

In anesthetized cats gastric motility was recorded by a volume method. Defined nociceptive physical stimuli (Dulcerogenic lesions or mechanical stimulation) promptly induced a pronounced gastric relaxation which persisted after administration of atropine and pentobarbitone as well as after adrenalectomy. The response was abolished by vagotomy or by spinal cord transection at the cervical level and was temporarily blocked by spinal anesthesia.

Afferent electric stimulation of cut mesenteric or splanchnic nerves also elicited gastric reflex relaxation with characteristics similar to that obtained on nociceptive stimulation.

It is concluded that nociceptive intestinal stimuli induce suppression of gastric motility via a reflex utilizing sympathetic afferents and ascending spinal pathways as the afferent link. The vagal non-adrenergic inhibitory system constitutes the efferent part of the reflex. The described intestino-gastric spino-vagal reflex is suggested to be important for the pronounced gastric inhibition in paralytic ileus.

Reference

WOLANSSON, H. H. GLISE and K. GLISE, *Scand J Gastroint* 1978, in press.

C 32

Characteristics of the Sympathetic Nervous Activity in Awake Normotensive and Hypertensive Rats

By S.-E. RICKSTEN and P. THORÉN, *Department of Physiology, University of Göteborg, Sweden*

The sympathetic nervous discharge was recorded from splanchnic branches in awake adult normotensive Wistar Kyoto WK (NCR) rats or spontaneously hypertensive rats SHR of the Okamoto strain.

During Nembutal® anaesthesia, small bipolar silver electrodes were placed on the sympathetic branch and isolated with Wacker silicone rubber (Sil Gel 604) according to the technique described by (Schad and Seller 1975). The animals were allowed to recover for at least 4 hours. Then the nervous activity, heart rate and blood pressure were recorded when the animals were fully awake. The normotensive rats showed at rest a very stable level of blood pressure, heart rate and nervous activity. In comparison SHR showed a much more varying level of sympathetic nervous activity. In both SHR and WK there was a close correlation between changes in sympathetic nervous activity and heart rate.

The reflex response in heart rate, blood pressure and nervous activity was examined

There is considerable morphological evidence that the rat myometrium receives both cholinergic and adrenergic innervation. However the function of these nerves is still largely unsettled. In the present communication we have studied some effects of transmural nerve stimulation in isolated uterine horns of rats.

Strips or segments of uterine horns under different hormonal conditions were placed in organ baths containing Mg^{2+} -free Ringer solution gassed with 5% CO_2 in O_2 , kept at $37^\circ C$. Spontaneous contractions and responses to transmural nerve stimulation (1–10 Hz, pulse duration 0.2–1.0 ms, supramaximal voltage) were recorded isotonically (load 0.5 g).

Transmural stimulation of uterine horns from nonpregnant rats produced inhibition of spontaneous activity whether the animals had received estrogen treatment for two weeks or not. This inhibitory response was annulled by tetrodotoxin ($0.3 \mu M$) and guanethidine ($1 \mu M$) but left unaffected by practolol ($2-4 \mu M$), phenoxybenzamine ($1-4 \mu M$) or phentolamine ($1-5 \mu M$). This suggests that the inhibitory response was due to stimulation of adrenergic nerves acting on β_2 -adrenoceptors.

Atropine ($0.3-1.0 \mu M$) did not significantly affect the spontaneous activity but often enhanced the inhibitory response, possibly due to removal of a cholinergic excitatory response. In order to unmask a cholinergic excitatory response the spontaneous activity had to be eliminated. This was achieved by either of the following treatments: 5–10 mM $MgCl_2$ and 5 mM $CaCl_2$ or 1 mM $MgCl_2$ at decreased bath temperature ($32^\circ C$) or inhibition of prostaglandin synthesis. After discontinuation of spontaneous contractions, transmural nerve stimulation of uterine horns from nonpregnant rats produced contractions that were blocked by tetrodotoxin ($0.3 \mu M$) or atropine ($0.3-1.0 \mu M$) but left unchanged by adrenergic blocking agents.

In contrast, transmural nerve stimulation of longitudinal strips from rats late in pregnancy (day 20–21) elicited spontaneous-like contractions whether the preparations were left spontaneously active or not. However these responses were unaffected by tetrodotoxin ($0.16-3.1 \mu M$) or drugs which block adrenergic as well as cholinergic receptors. The absence of nerve induced uterine responses in the 20–21 day pregnant rat, may at least in part be explained by the reduction of innervation density that has been reported late in pregnancy.

Our study indicates that the non-pregnant rat uterus has both cholinergic excitatory and adrenergic inhibitory responses which however seem to disappear late in pregnancy.

C 31

Vagal Inhibition of Gastric Motility Elicited from the Small Intestine

By H. ABRAHAMSSON and H. GLISE. *Department of Physiology, University of Göteborg and Department of Medicine II, Sahlgren's Hospital, Sweden*

Gastrointestinal motility is greatly impaired by intraabdominal nociceptive stimulation e.g. abdominal surgery. An increased adrenergic nervous activity is commonly regarded as the main pathogenetic factor in such paralytic ileus. However, in a recent study

(Abrahamson, Glise and Glise 1978) nociceptive stimuli in the gastroduodenal area was found to suppress gastric motility by activation of vagal non-adrenergic inhibitory fibres to the stomach. The present study was designed to explore the possible reflex pathway for vagal gastric inhibition in connection with nociceptive stimuli in the small intestine.

In anesthetized cats gastric motility was recorded by a volume method. Defined nociceptive jejunal stimuli (Diathermic lesions or mechanical stimulation) promptly induced a pronounced gastric relaxation which persisted after administration of atropine and pentethidine as well as after adrenalectomy. The response was abolished by vagotomy or by spinal cord transection at the cervical level and was temporary blocked by spinal anesthesia.

Afferent electric stimulation of cut mesenteric or splanchnic nerves also elicited gastric reflex relaxation with characteristics similar to that obtained on nociceptive stimulation.

It is concluded that nociceptive intestinal stimuli induce suppression of gastric motility as a reflex utilizing sympathetic afferents and ascending spinal pathways as the afferent link. The vagal non-adrenergic inhibitory system constitutes the efferent part of the reflex. The described testino-gastric spino-vagal reflex is suggested to be important for the produced gastric inhibition in paralytic ileus.

Reference

ABRAHAMSON H, H GLISE and K. GLISE. *Scand J Gastroenter* 1978. In press

C 32

Characteristics of the Sympathetic Nervous Activity in Awake Normotensive and Hypertensive Rats

By S E RICKSTEN and P THOREN. *Department of Physiology, University of Göteborg, Sweden*

The sympathetic nervous discharge was recorded from splanchnic branches in awake adult normotensive Wistar Kyoto WK (NCR) rats or spontaneously hypertensive rats SHR of the Okamoto strain.

During Nembutal® anaesthesia, small bipolar silver electrodes were placed on the sympathetic branch and isolated with Wacker silicone rubber (Sil Gel 604) according to the technique described by (Schad and Seller 1975). The animals were allowed to recover for at least 24 hours. Then the nervous activity, heart rate and blood pressure were recorded when the animals were fully awake. The normotensive rats showed at rest a very stable level of blood pressure, heart rate and nervous activity. In comparison SHR showed a much more varying level of sympathetic nervous activity. In both SHR and WK there were a close correlation between changes in sympathetic nervous activity and heart rate.

The reflex response in heart rate, blood pressure and nervous activity was examined.

during blood volume expansion Upon 10% increase in blood volume the splanchnic nervous activity decreased 42% in SHR and 33% in NCR The same transfusion increased blood pressure 5.1 mmHg in SHR and 9.7 mmHg in NCR Thus upon volume load the hypertensive animals seem to increase the blood pressure less in comparison with WK because the sympathetic nervous activity is decreased more in SHR than in NCR The only reasonable explanation for this finding is a hyperreactive volume receptor mechanism in the spontaneously hypertensive rats

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Reference

SCHAD H and H SELLER, *Brain Res* 1975 100 425-430

C 33

Diurnal Variation of Immunoreactive LRH Concentrations in Hypothalamus Preoptic Area and Amygdala in Male Rats

By M TUOMINEN H SELANNE and J LEPPÄLUOTO *Department of Physiology University of Oulu Finland*

Serum LH levels show random fluctuation but no clear circadian rhythm (Kaira and Kaira 1977) This would indicate that hypothalamic LRH concentrations do not undergo distinct circadian rhythm Therefore we have measured hypothalamic LRH concentrations at various time of day Because of conflicting reports about the effects of electrical and chemical stimuli to amygdala on gonadotropin release we also measured the LRH concentrations of amygdala

Male rats were housed for 3 weeks in a silent room with lights on from 8 AM to 6 PM Five or six rats were rapidly decapitated at 4 hrs intervals The tissue samples were excised extracted with methanol evaporated dissolved in buffer and measured in a specific radioimmunoassay for LRH (Seppälä, Ranta and Leppäluoto 1974) Hypothalamic samples included the median eminence and its surrounding between mamillary bodies and optic chiasma and they weighed from 8 to 16 mg Preoptic areas weighed from 5 to 11 mg and amygdalae from 17 to 25 mg.

Hypothalamic and preoptic LRH concentrations did not show any statistical variations either in the light or dark period of the day Hypothalamic LRH concentrations varied from 646 to 970 pg per mg of wet tissue and preoptic ones from 70 to 160 pg/mg The highest LRH concentrations in amygdala was found at 4 PM 11.5 ± 1.8 pg/mg and the lowest at 4 AM 2.3 ± 1.5 pg/mg ($p > 0.01$ to 4 PM values)

The absence of circadian rhythm either in hypothalamic or preoptic LRH concentrations in male rats agrees with the findings of Kaira and Kaira (1976) Further our finding of

diurnal fluctuation of LH concentration in amygdala might be in some relation with that of FSH in serum (Kaira and Kaira 1977). This result does not conflict with the finding that stimulation of amygdala leads to gonadotropin release (Kawakami & Terasawa 1974).

References

- KAIRA, P. S. and S. P. KAIRA, *Endocrinology* 1977 101 1821
 KAWAKAMI, M. and E. TERASAWA, *Biological Rhythms in Neuroendocrine Activity* pp. 197-219 1974
 SERRA, M., T. RANTA and J. LEPPÄLÖTTÖ, In: *Biological and clinical aspects of reproduction* Excerpta Medica International Congress Series No. 394 Excerpta Medica, Amsterdam 1974 463-471

C 34

The Effect of Norepinephrine on Hippocampal Pyramidal Cells *in vitro*

by L. A. LANGVORN and M. SEGAL, *Institute of Neurophysiology University of Oslo Norway*

Extracellular recordings from hippocampal pyramidal cells have suggested that norepinephrine (NE) is a putative inhibitory transmitter in the hippocampus (Segal and Bloom, 1974). The mechanism for this inhibition, however, is still unknown. The aim of the present study was to investigate by intracellular recordings the action of NE on the hippocampal pyramidal cells.

Recordings were obtained from CA1 pyramidal cells using hippocampal slices maintained *in vivo* (Stredje and Westgaard 1971). Prior to the application of NE, control responses to depolarizing and hyperpolarizing current pulses passed through the recording electrode were obtained for 1-8 minutes. Droplets (ca. 1 nanoliter) of NE (10^{-8} - 10^{-6} M) were then applied from a pipette positioned close to the recording electrode.

After application of NE the cell hyperpolarized up to 4-5 mV accompanied by an up to 25-30% reduction in resistance. The two effects were always closely associated with a reduction in the number of spikes elicited by depolarizing current pulses. In spontaneously firing cells NE either reduced the firing rate or completely abolished spontaneous activity. The resting potential, membrane resistance and spontaneous and evoked activity all recovered within a period of 10 minutes after application. Similar results were obtained in preliminary experiments in which NE was administered iontophoretically.

In order to exclude the possibility that the inhibitory effect of NE was mediated through interneurons, applications of NE were made after reducing calcium and increasing magnesium levels in the incubation solution. While this procedure completely blocked synaptic transmission NE still had similar effects on the pyramidal cells.

Our results support the concept that NE serves as an inhibitory transmitter on the CA1 hippocampal pyramidal cells. The mechanism for this inhibition seems to be a combination of increased conductance and hyperpolarization of the cell membrane.

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References

- SEGAL, M. and F. E. BLOOM *Brain Res.* 1974 72 79-97
 SEGAL, M. and F. E. BLOOM *Brain Res.* 1974 72 99-114
 SKREDE, K. K. and R. H. WESTGAARD *Brain Res.* 1971 35 589-593

C 35

Synaptic Connections to Perigeniculate Neurones

By GUNILLA AHLSEN and SIVERT LINDSTRÖM *Department of Physiology, University of Göteborg, Göteborg, Sweden*

Postsynaptic inhibition can be evoked in principal cells of the lateral geniculate body by electrical stimulation of the primary visual cortex. It has been suggested that this type of inhibition is mediated by a subcortical recurrent inhibitory pathway involving axon collaterals of principal cells and an inhibitory interneurone (Andersen, Eccles and Sears 1964, Burke and Sefton 1966). In keeping with this idea, it has recently been found that principal cells issue axon collaterals dorsally to the geniculate body in the perigeniculate region (Ahlén, Lindström and Sybirska 1978). Neurones in this region have now been studied to determine if they might serve as interneurons in the suggested recurrent pathway to principal cells.

The experiments were performed on adult cats under pentobarbital anaesthesia. Stimulation electrodes were placed in the optic tract and in the primary visual cortex. Extra- and intracellular recordings have been obtained from 38 perigeniculate neurones identified by their location 100-400 μm above the lateral geniculate body. The cells were synaptically activated by stimulation of the optic tract and the visual cortex. Usually a train of spikes could be evoked by both types of stimulation. The latency of the first spike indicates a monosynaptic linkage from the cortex and a disynaptic linkage from the optic tract as would be expected if the cells were excited via axon collaterals of principal cells. Collision tests performed with a few intracellularly recorded cells have confirmed that the first part of the excitation is mediated by such collaterals.

The later spikes in the train seem to be due to a different excitatory component which presumably is mediated by slowly conducting corticofugal fibres. In all tested cells the excitation was followed by a longlasting inhibition both when the cortex and the optic tract were stimulated. The properties of this inhibition indicates that it is mediated by mutual inhibitory connections between perigeniculate neurones. All these synaptic effects on perigeniculate neurones closely match the behavior of the inhibitory potentials evoked in principal cells by similar stimulations. Thus, the present observations give strong support to the hypothesis that perigeniculate neurones are interposed as interneurons in a recurrent inhibitory pathway to principal cells.

References

- AHLÉN, G., S. LINDSTRÖM and E. SYBIRSKA *Brain Res.* 1978 In press.
 ANDERSEN, P., J. C. ECCLES and T. A. SEARS, *J. Physiol.* 1964 174 370-399
 BURKE, W. and A. J. SEFTON *J. Physiol.* 1966 187 231-246.

C 36

Motor Activity Retards Kindling Rate in Rats

By SVEN GRAHNQVIST *Institute of Physiology University of Bergen Norway*

Several reports have suggested that increased CNS activation is inhibitory to the occurrence of epileptic seizures. It has also been shown that reduced stress and thus possibly reduced activation, will facilitate the development of epileptic susceptibility. In this experiment, activation expressed as motor activity vs inactivity was investigated regarding effects on development of epileptic susceptibility.

Repeated electrical stimulation of the brain with low current intensities leads to a progressive decrease in seizure threshold. Goddard named this process kindling in his original report. The kindling paradigm provides an excellent model of epilepsy that is easy to control, and interference with normal behavior is minimal.

For this experiment rats were implanted with bipolar electrodes in the right amygdala. All animals were handled every day from one week before surgery till testing started two weeks after surgery. The animals were kept on a 12 hour light-dark cycle in a sound-shielded room to minimize external interference with the experiment. Each day all animals were allowed at least three minutes in the testing chamber for habituation and scoring of spontaneous activity before stimulation. After having shown the desired activity for at least three seconds, the rats were stimulated.

Seizure development was scored according to Racine. Number of days of stimulation before showing a class 5 seizure—that is a full motor seizure with loss of equilibrium—was recorded. The rats stimulated when showing motor activity were significantly slower than those stimulated when quiet, in reaching criteria.

This experiment has shown that normal behavior can influence the development of epileptic susceptibility in rats. In particular motor activity retarded the kindling rate. The results are in agreement with earlier findings that has shown that increased activation is inhibitory to development of epileptic susceptibility as well as occurrence of seizures in susceptible individuals.

References

- ANGOLD, P. S., R. J. RACINE and R. A. WISE, *Exp Neurol* 1973 40 457-470.
 GODDARD, G. V., D. C. MCINTYRE and C. K. LEBER, *Exp Neurol* 1969 25 295-330.
 PHILL, J. B., J. A. PHILLIPS and B. MACNEILL, *Epilepsia* 1973 14 29-37.
 RACINE, R. J. *Electroencephalogr and Neurophysiol* 1972 32 281-294.

References

- SEGAL, M and F E BLOOM *Brain Res* 1974 72 79-97
 SEGAL, M and F E BLOOM *Brain Res* 1974 72 99-114
 SKREDE K KR and R H WESTGAARD *Brain Res* 1971 35 589-593

C 35

Synaptic Connections to Perigeniculate Neurones

By GUNILLA AHLSEN and SIVERT LINDSTRÖM *Department of Physiology University of Göteborg Göteborg Sweden*

Postsynaptic inhibition can be evoked in principal cells of the lateral geniculate body by electrical stimulation of the primary visual cortex. It has been suggested that this type of inhibition is mediated by a subcortical recurrent inhibitory pathway involving axon collaterals of principal cells and an inhibitory interneurone (Andersen, Eccles and Sears 1964, Burke and Sefton 1966). In keeping with this idea, it has recently been found that principal cells issue axon collaterals dorsally to the geniculate body in the perigeniculate region (Ahlén, Lindström and Sybirska 1978). Neurones in this region have now been studied to determine if they might serve as interneurones in the suggested recurrent pathway to principal cells.

The experiments were performed on adult cats under pentobarbital anaesthesia. Stimulation electrodes were placed in the optic tract and in the primary visual cortex. Extra- and intracellular recordings have been obtained from 38 perigeniculate neurones identified by their location 100-400 μ m above the lateral geniculate body. The cells were synaptically activated by stimulation of the optic tract and the visual cortex. Usually a train of spikes could be evoked by both types of stimulation. The latency of the first spike indicates a monosynaptic linkage from the cortex and a disynaptic linkage from the optic tract as would be expected if the cells were excited via axon collaterals of principal cells. Collision tests performed with a few intracellularly recorded cells have confirmed that the first part of the excitation is mediated by such collaterals.

The later spikes in the train seems to be due to a different excitatory component which presumably is mediated by slowly conducting corticofugal fibres. In all tested cells the excitation was followed by a longlasting inhibition both when the cortex and the optic tract were stimulated. The properties of this inhibition indicates that it is mediated by mutual inhibitory connections between perigeniculate neurones. All these synaptic effects on perigeniculate neurones closely match the behavior of the inhibitory potentials evoked in principal cells by similar stimulations. Thus the present observations give strong support to the hypothesis that perigeniculate neurones are interposed as interneurones in a recurrent inhibitory pathway to principal cells.

References

- AHLSEN G S LINDSTRÖM and E. SYBIRSKA *Brain Res* 1978 In press.
 ANDERSEN P J C ECCLES and T A SEARS *J Physiol* 1964 174 370-399
 BURKE, W and A J SEFTON *J Physiol* 1966 187 231-246

It is concluded that a decrease in gall-bladder transepithelial net Na-transport of about 50% induced by a small serosal hydrostatic pressure of 4.5 cm water results mainly from a decrease in J_{Na} , and not from an increase in paracellular leakage. Thus the present results support the hypothesis that gall-bladder transepithelial Na-transport is determined by mechanical forces.

References

- DIAMOND, J. M. and W. H. BOSSERT *J gen Physiol* 1967 50 2061-2083
FERNBERG, O. and P. P. LEVETZAC, *J Physiol (London)* 1969 201 201-224

D 2

Effects of "Physiological" Concentrations of Adrenaline on Perfused Fish Gills

By I. WAHLQVIST and S. NILSSON *Department of Zoophysiology, University of Göteborg, Sweden*

In teleost fish, catecholamines are stored in and released from chromaffin tissue in the anterior part of the kidney (head kidney). Stress produced by keeping the animal in air for 15 min, induces a release of amines and the plasma concentration of adrenaline increases from 0.04 to 0.23 μM in blood sampled from the caudal vessels (Nilsson *et al* 1976). New preliminary results suggest that the plasma concentration in blood sampled from a gill artery, i.e. closer to the source of adrenaline release, might be even higher.

These changes in concentration of adrenaline in plasma may affect the contractile force of the cod heart. $\text{EC}_{50} = 0.23 \mu\text{M}$ for the positive inotropic effect of adrenaline on the cod



Fig. 1 Increase in the rate of flow through isolated perfused cod gill arches produced by increasing concentrations of adrenaline. The shaded area indicates the "physiological" range of adrenaline concentration in plasma from blood sampled in the caudal vessels of non-stressed and stressed fish. Number of estimations in each point = 10-14, variation shown as standard error (S.E.).

DEMONSTRATIONS

D 1

Sensitivity of Gall-bladder Na-transport to a small Serosal Hydrostatic Pressure

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Two fundamentally different hypotheses have been proposed to explain transepithelial transport of Na salts in isosmotically transporting epithelia such as the gall-bladder. The one postulates Na-transport by an ion- or salt pump localized in the lateral cell membrane; a resulting local hypertonicity in the lateral intercellular space is held to be responsible for consequent passive osmotic transport of water (Diamond & Bossert 1967). According to the alternative hypothesis transcellular isosmotic transport proceeds by transport of Na-salts and water as fluid volumes in separate subcellular compartments by means of mechanical (contractile) forces (Fredenksen & Leyssac 1969). The former theory predicts that active Na-transport should be rather insensitive to small serosal hydrostatic pressures, while the latter may allow a very high sensitivity of the active Na transport to such pressures. Thus a distinction between the two concepts might be possible from estimates of the pressure sensitivity of the mucosal-to-serosal Na-flux (J_{ms}^{Na}) and the passive serosal-to-mucosal Na-backflux (J_{sw}^{Na}) provided that gross leakage of the epithelium due to serosal pressure elevation can be avoided.

In the present investigation rabbit gall bladders were mounted as flat sheets in Ussing chambers. A thin soft nylonnet supported the mucosal surface. Transepithelial potential difference (P.d.), Resistance (R_T) and J_{ms}^{Na} or J_{sw}^{Na} (as ^{22}Na flux) was determined. A serosal pressure of 4.5 cm water was applied for 40 min by elevating the serosal half-chamber. Unidirectional Na-fluxes are in terms of $\text{neq} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$. All data are given as means \pm SEM.

The serosal pressure caused J_{ms}^{Na} to decrease from 453 ± 6 to 233 ± 15 ($n=6$); the half-time was 6 min. In the recovery period J_{ms}^{Na} returned to 456 ± 77 ; the half-time was 4.5 min. J_{sw}^{Na} ($n=6$) increased transiently within 5 min of pressure application from 137 ± 7 to 308 ± 38 and then decreased rapidly again to near control values; after 40 min J_{sw}^{Na} was 177 ± 9 and in the recovery period 132 ± 9 . The serosal pressure caused R_T to increase from 63.7 ± 3.8 to 80.3 ± 2.9 $\text{ohm} \cdot \text{cm}^2$ ($n=17$). After 40 min recovery R_T was 66.8 ± 4.1 $\text{ohm} \cdot \text{cm}^2$. P.d. showed only small changes: typically a reversible decrease of about 0.5 mV from control values of 3 mV (mucosa positive) was observed during pressure application.

rats of group A and C were further unilaterally sympathectomized by surgical extirpation of the sympathetic chain from above the 2nd lumbar to below the 2nd sacral ganglion. The rats of group D were left intact (sham-operated). Sympathectomy (with or without adrenodemedullation) results in a 90% reduction in the noradrenaline content of hind limb muscles (Richter, Galbo and Christensen 1978). Half of the rats in each group (T-rats) were subjected to a 12 week physical training programme consisting of swimming in water maintained at 33–34°C, up to 5.5 hours/day 4 days/week. The remaining rats served as sedentary controls (C-rats). 72 hours after the last training session, the rats were weighed and the gastrocnemius muscles were excised bilaterally under pentobarbital anaesthesia and immediately frozen in liquid nitrogen for subsequent analysis of succinate dehydrogenase (SDH, EC 1.3.99.1) activity as an indicator of oxidative potential.

Irrespective of the operative treatment the weight of the gastrocnemius muscle was lower in T-rats opposed to C-rats (-13% , $p<0.001$). However gastrocnemius weight relative to body weight were equal for all rats. The SDH activities for the differently treated hind limbs are as follows ($\mu\text{mol} \times \text{g}^{-1} \text{ wet weight} \times \text{min}^{-1}$) (means \pm SE): Shamoperated 11.9 ± 0.4 (T_{sh}) vs. 8.1 ± 0.3 (C_{sh}) $+46\%$, $p<0.001$; Intact adrenal medulla sympathectomized 9.6 ± 0.6 (T_{ad}) vs. 7.2 ± 0.5 (C_{ad}) $+33\%$, $p<0.01$; adrenodemedullated intact sympathetic chain 10.6 ± 0.4 (T_{ad}) vs. 7.8 ± 0.3 (C_{ad}) $+35\%$, $p<0.001$ and adrenodemedullated sympathectomized 10.4 ± 0.6 (T_{ad}) vs. 7.4 ± 0.6 (C_{ad}) $+45\%$, $p<0.01$.

Although a tendency towards a somewhat greater increase in SDH activity in the shamoperated rats can be observed it is concluded that an intact sympatho-adrenal system is not necessary for a marked oxidative enzymatic adaptation of skeletal muscle to increased activity to take place.

References

- FLUKE, M. N. E. *Med Biol* 1977 55: 268–276.
 FLUKE, M. N. E. and J. VALTOLA, *Acta physiol scand* 1975 95: 391–399.
 RICHTER, E. A., H. GALBO and N. J. CHRISTENSEN, *Acta physiol scand* 1978. This suppl.

D 4

Correlation Between Tension and Structure in a Smooth Muscle During Phasic Contractions
 By KELD HORNBECH SVENDSEN *Institute of Biophysics University of Aarhus Aarhus Denmark*

The structure of smooth muscles is still relatively unknown and in published works the muscles have only been investigated under steady state conditions (Vibert et al. 1972). The purpose of the work reported here has been to give information about structural changes in a smooth muscle during phasic contractions where the tension show both contracting and relaxed phases.

atrum (Holmgren 1977) There are also some indirect indications that circulating catecholamines may contribute to the overall control of blood pressure in the cod (Wahlqvist and Nilsson 1977)

The vascular resistance in teleost gills is in contrast to the systemic resistance reduced by catecholamines (for references see e.g. Wood 1974) The present study was undertaken to examine the possible control of the branchial vasculature by "physiological" plasma concentrations of adrenaline

Isolated gill arches from Atlantic cod *Gadus morhua* were perfused at a constant pressure with a cod Ringer's solution containing increasing concentrations of adrenaline. Adrenaline produced a biphasic change in flow rate: a small initial decrease was followed by an increase in perfusion rate. The initial decrease can be abolished by phentolamine $1 \mu\text{M}$

Concentrations of adrenaline similar to those measured in plasma during "stress" may have pronounced effects on the vascular resistance of cod gills (Fig. 1). The range of adrenaline concentrations in "non-stressed" and "stressed" fish could be even higher than indicated by the figure with higher levels of adrenaline close to the head kidney. The possibility of a considerably lower plasma concentration of adrenaline in "non-stressed" fish under normal non-laboratory conditions must also be remembered

References

- HOLMGREN S. *Acta physiol scand* 1977 99 6-74
 NILSSON S. T. ABRAHAMSSON and D. J. GROVE. *Comp Biochem Physiol* 1976 55C 123-127
 WAHLQVIST I. and S. NILSSON. *Comp Biochem Physiol* 1977 57C 65-67
 WOOD C. M. *J Exp Biol* 1974 60 241-265

D 3

Significance of the Sympatho-adrenal System for the Exercise-Induced Enzymatic Adaptation of Skeletal Muscle

By J. HENRIKSSON, J. SVEDENHAG, E. A. RICHTER and H. GALBO. Department of Physiology III, Karolinska Institutet, Stockholm, Sweden and the Department of Medical Physiology b, University of Copenhagen, Denmark

The stimuli triggering the adaptive changes in skeletal muscle oxidative potential to different levels of physical activity are unknown. Based on studies with daily injections of isoprenaline as well as with prolonged beta-blockade in rats (Harr and Valtola 1975, Harr 1977) the idea has been put forward that specific activation of beta-receptors on the muscle fibres trigger the enzymatic adaptation. The purpose of the present study was to further evaluate this possibility.

88 male Sprague-Dawley rats (130 g) were randomly divided into four groups (A-D). In group A and B the rats were adrenomedullated bilaterally by electrocoagulation. The

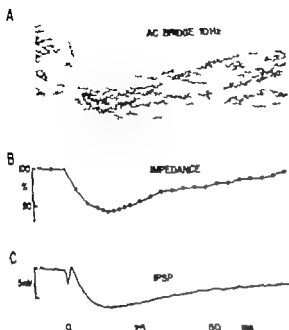


Fig. 1 A. 35 superimposed sweeps of the voltage deflections resulting from 0.15 nA 10 Hz AC current through the recording electrode during the IPSP B. Relative impedance of the cell, as measured from part A. Plotted in percent of resting impedance C. The timecourse of the IPSP

The conductance change underlying the IPSP was studied by sending a 10 Hz AC current through the intracellular recording electrode. By superimposing the records from 20 to 50 such sweeps a picture as the one shown in Fig. 1 A was obtained. From such pictures the impedance was measured in per cent of resting impedance.

There seemed not to be any big difference in the time course of the IPSP and in the impedance change. In the cell presented in Fig. 1 both the conductance increase and the IPSP were largest after 15 ms. Similar results were obtained from other cells with the peak in conductance however usually 3–4 ms before the maximal amplitude of the IPSP. When the cells were hyperpolarized to the equilibrium potential of the IPSP orthodromically evoked EPSP's were only shunted during the period of largest conductance increase (up to 15–25 ms). The probability of firing on orthodromic stimulation could however be reduced for a longer period (up to 60 ms).

The hyperpolarization and the conductance change in recurrent inhibition on hippocampal pyramidal cells show similar time course. Our data suggest that the hyperpolarization and the conductance increase cooperatively inhibit the cell during the period of largest conductance change. In the later part of the IPSP however inhibition seems to be produced mainly by the hyperpolarization.

References

- A. DERSI, P. J. C. ECCLES and Y. LÖNNING, *J. Neurophysiol.* 1964 27 392–407
 ANDERSEN, P. J. C. ECCLES and Y. LÖNNING, *J. Neurophysiol.* 1964 27 608–619
 SCALDE, K. KR. and R. H. WESTGAARD, *Brain Res.* 1971 35 589–595

After dissection the ABRM muscle from *Mytilus Edulis* was placed in seawater containing 5-HT. For activation a 7 s electrical stimulation was used (Cornelius and Lowy 1978).

The x ray diffraction method gives a diffraction pattern with two prominent reflections, namely the meridional 144 Å reflection from the periodic structure of myosin filaments and the intensity distribution along the equatorial direction from the planar arrangement of actin filaments (e.g. 2-dimensional electron density projected in a plane at right angle to the longitudinal axis of the muscle). The innermost part of the equatorial distribution provides information about variations in the electron density at the outer part of the actin filaments, e.g. decoration with HMM-S1 but is relatively insensitive to changes in the actin filament packing.

The observations show that while development of maximum tension and the following relaxation each takes about 10 s, the change of structure takes place during the very first second and after the stimulation is finished it takes 30–40 s before the structure returns to a relaxed state. The experiments also showed that the diffracted intensity was insensitive to transitions between phasic contracting and tonic states. If no 5-HT was added to the seawater the resting state could give diffracted intensities similar to those obtained from contracted states. My interpretation is that a constant fraction of HMM-S1 molecules can be attached to actin filaments even if their contribution to the developed tension are varying.

References

- VIBERT P. J., HASELGROVE J. R., LOWY J. and P. R. POULSEN *J. Mol. Biol.* 1977, 71, 757–767.
 CORNELIUS F. and J. LOWY *Acta physiol. scand.* 1978, 102, 167–180.

D 5

On the Time-course of Recurrent Inhibition in Hippocampal Pyramidal Cells *in vitro*

By I. A. LANGMOEN and R. DINGLEDINE *Institute of Neurophysiology, University of Oslo, Norway*

Recurrent inhibition on the hippocampal pyramidal cells is produced by activation of basket cells which have their synapses on the somata of pyramidal cells (Andersen, Eccles and Loynning, 1964). Synaptic inhibition is supposed to work through a hyperpolarization of the cell membrane as well as a conductance increase of the cell membrane which inhibits loading of its capacitative elements.

In hippocampal pyramidal cells there are no data available comparing the hyperpolarization with the underlying conductance change. The aim of the present study was to study the time course of the two events and investigate which of the factors plays the most important role in the inhibition of the spike discharges.

Intracellular recordings were obtained from the hippocampal pyramidal cells *in vitro* (Skrede and Westgaard 1971). IPSPs were evoked by subthreshold antidromic stimulation

farther away from the tooth and its supporting tissues (Linden 1978). Recent studies in cats have demonstrated that the discharge from the intraoral mechanoreceptors can be reduced by electrical stimulation of sympathetic fibres to the area (Anderson and Linden 1977). This effect could result from either movement of the tooth secondary to vascular clamps (Aars 1978) or to more direct influences on the receptors. As a first step to studying these mechanisms, we have investigated the effect of electrical stimulation of the cat cervical sympathetic nerve on longitudinal and transverse movements of the canine tooth.

The cats were anaesthetized with sodium pentobarbitone and tracheotomized. Blood pressure was recorded in a femoral artery and body temperature was maintained using a thermostatically controlled blanket. Longitudinal and labial/palatal movements of the left upper canine were determined by ultrasonic transit time technique (Leraand 1970) with one set of crystals fixed to the tooth, the other in the maxilla or to a frame supporting the head.

In five of six cats the canine intruded a maximum of 5–15 μm during sympathetic stimulation (5 V 1 ms 1 20 Hz 30 s periods): extrusion was seen in one. Three canines moved in a labial direction, while the others responded with a palatal movement. These longitudinal and transverse movements were usually evoked by stimulation of high-threshold fibres and could be blocked by phentolamine (2–4 mg/kg). In only one cat, excitation of low-threshold (1.5 V 0.02 ms) fibres in the sympathetic nerve induced intrusion and a palatal movement which was blocked by atropine 0.5 mg/kg, whereas activity in the higher threshold fibres evoked intrusion and a post-stimulatory palatal movement. In another cat, a 70 Hz 1 ms stimulation caused a labial movement at 2.5 V and a palatal movement at 5 V.

Our results demonstrate that the cat canine will move in response to sympathetic nerve stimulation and suggest that the movements are mediated mostly by adrenergic more rarely by cholinergic vascular receptors. The direction and extent of movement differs from one cat to another and can be complex.

References

- AARS, H. *Acta physiol scand* 1978 102 34–35A.
 ANDERSON, D. J. A. and R. W. A. LINDEN. *J dent Res Special Issue* 1977 56, D125.
 LERAAND, B. *Proc 1st Nordic Meeting on Med. & Biol. Eng.* 1970 187–190.
 LINDEN, R. W. A. *J Physiol* 1978 279 395–406.

D 6

Mitochondria in Rat Intestinal Epithelium

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Because of their high turnover rate (48 h in the rat) the intestinal epithelial cells provide an interesting system for the regulation research of the energy metabolism.

D 6

Spatial Analysis of the Freeze Trapped Brain Provides for Temporal Resolution of an Event. Metabolic Electrical and Blood Flow Changes During Spreading Depression

By B. QUISTORFF, A. GJEDDE and A. J. HANSEN. *Department of Biochemistry A and Institute of Medical Physiology, Dept. A, Panum Institute, Copenhagen, Denmark.*

Spreading depression (SD) has been studied in terms of changes of energy metabolism, blood flow and extracellular potassium concentration (K_e). The SD wave was elicited in nembutal anesthetized, craniotomized rats by means of mechanical stimulation in the left frontal cortex and the progression of the wave was followed by a number of suitably spaced cortical electrodes recording either K_e or DC potential. The brain was freeze trapped during the progression of the SD wave by funnel freezing with liquid nitrogen. Prior to freezing, a bolus of ^{14}C butanol was injected in a femoral vein and blood was sampled continuously during a 10 sec period until moment of freezing. The frozen brain was sectioned in coronal slices of a thickness of 1.2 mm. Right and left side cortex in these slices were dissected at $-20^\circ C$ and extracted with Lowry-technique for measurements of PCr, ATP, ADP, AMP, lactate and pyruvate. ^{14}C activity was measured for calculation of regional blood flow.

Assuming no regional differences in the rat brain cortex with respect to response to the SD wave, the spatial analysis of the freeze trapped brain gives a temporal resolution of the SD wave. The results may be summarized as follows: The change of K_e during the SD wave lasts approximately 1 min, with a sharp rise demarcating the wave front, followed by a plateau and then a gradual decline to normal K_e . Related to this time scale, changes observed in regional metabolite concentrations and blood flow have a wider extension. Expressed as % of the contralateral region of the unaffected cortex, there is a flow decrease of 10–15% immediately preceding the SD wave, of an extension of about 1 min, coinciding with a decrease in the $\frac{ATP}{ADP}$ ratio (10%) as well as a 100% increase in the lactate/pyruvate ratio. The sharp rise in potassium is accompanied by a massive increase in blood flow (100–150%) now coinciding with a normalization of the ATP/ADP ratio and an abrupt 5-fold decrease in lactate/pyruvate ratio. The flow changes as well as the redox changes normalize somewhat slower than the extracellular potassium concentration.

D 7

Movements of the Cat Canine Tooth in Response to Sympathetic Nerve Stimulation

By R. W. A. LINDEN and H. AARS. *Department of Physiology, Guy's Hospital Medical School, London, England and Department of Physiology and Biochemistry, Dental Faculty, University of Oslo, Norway.*

Activity of intraoral mechanoreceptors that respond to forces applied to teeth are relayed to the brain stem through trigeminal afferent fibres. These receptors include both periodontal mechanoreceptors and "Type P" mechanoreceptors whose site is thought to be some

The effect of intracranial stimulation of the seventh cranial nerve (n. facialis-n. intermedius) on the blood flow of the eye and the tongue was studied in rabbits. The nerve was electrically stimulated with a stereotaxic technique: pulse intensity 6–14 V, duration 1 sec, and frequency 40–50 Hz. Regional blood flow in the eye and the tongue was measured with radioactively labelled microspheres.

Stimulation caused a $212 \pm 39\%$ ($n=18$) ($p<0.001$) increase in choroidal blood flow on the stimulated side as compared to the control side. Flow rates in the ciliary processes and the iris increased by $53 \pm 19\%$ ($n=18$) ($p<0.02$) and $11 \pm 5\%$ ($n=18$) ($p<0.001$) respectively. Lingual blood flow also tended to increase during stimulation. The blood flow of the mucous membrane of the anterior part of the tongue increased by $210 \pm 64\%$ ($n=17$) ($p<0.01$) on the stimulated side. The blood flow of the corresponding muscle layer of the tongue also increased but with a great variability in the results. The duration of the vasodilation of the eye after a period of stimulation was 30–60 min while in the tongue it was about 10 min. Neither the increase in blood flow of the eye nor that of the tongue could adequately be abolished by cholinergic blockade with biperiden (5–10 mg/kg b.w.) or by peptone blockade with hexamethonium (50–100 mg/kg b.w.). Transection of the trigeminal nerve prior to stimulation of the facial nerve did not prevent the vasodilation.

The phenomenon of atropine-resistant vasodilation in the tongue as a result of corda trapezi stimulation is well-known and recent studies by Hellekant (1977) indicate that it is due mainly to activation of nerves originating in the CNS and not in the geniculate ganglion, that is to nerves which seem to be efferent. It is also known that close arterial injection of acetylcholine causes marked but short lasting vasodilation in the choroid in rabbits (Bull 1962).

The efferent autonomic nerves known so far in the Intermedio-facial nerve are cholinergic and have synapses in the pterygopalatine or submandibular ganglia. Such efferent nerves cannot explain the present results. The results rather indicate that the intracranial part of the Intermedio-facial nerve contains unconventional efferent or afferent fibres that on stimulation release a vasodilating substance in the eye and the tongue that is not acetylcholine.

References

- HELLEKANT T. *Acta Physiol Scand* 1977 99 292–299
BULL A. *Acta Physiol Scand* 1962 36 70–82.

D 10

Turnover of Noradrenaline, Dopamine and 5-Hydroxytryptamine in the CNS under the influence of 4-Antipyrindine

By NILS-ERIK ANDÉN and STEFAN LEANDER, Departments of Pharmacology, Universities of Göteborg and Lund, Sweden

4-Antipyrindine (4-AP) enhances nerve impulse evoked release of both acetylcholine and noradrenaline from peripheral nerves (Leander et al. 1977, Lundh et al. 1977). 4-AP also

TABLE I Protein yield cytochrome concentrations succinate dehydrogenase activity and oxygen consumption using succinate as a substrate in mitochondria of intestinal epithelial cells. Values are mean values \pm S.E. from 12-14 determinations

Protein yield mg/100 g body wt	7 \pm 0.57
Cytochrome aa ₃ nmol/mg protein	0.29 \pm 0.02
Cytochrome b nmol/mg protein	28 \pm 0.02
Cytochromes c+c nmol/mg protein	0.38 \pm 0.03
Cytochrome aa ₃ : Cytochromes c+c	1:1.3
Succinate dehydrogenase μ mol/min/mg protein	
Actual	0.22 \pm 0.02
Maximal	0.55 \pm 0.03
Oxygen consumption natoml/min/mg protein	
State 4	18.9 \pm 5.3
State 3	25.3 \pm 2.1
Respiratory control ratio State 3/State 4	1.4 \pm 0.06

In the present preliminary work conditions of the isolation of rat intestinal epithelial mitochondria and their general properties were determined. Intestinal epithelial cells of Sprague-Dawley rats were separated principally according to Harrison and Webster (1969) but using 0.3 M mannitol—10 mM Tris—5 mM EDTA—3 mM MgCl solution pH 7.4. The cells were sedimented and intestinal mitochondria were isolated using the same medium. Mitochondrial cytochromes were determined in 1 M sucrose at liquid nitrogen temperature (Wilson 1967). Absorption coefficients of De Jonge and Hulsman (1973) were used for calculating the cytochrome concentrations. Both actual and maximum activity of succinate dehydrogenase were determined by the method of King (1967) by using substrate activation (Kimura *et al.* 1967). Mitochondrial oxygen consumption was determined using a Clark type oxygen electrode. The results are represented in Table I.

The present results also showed that if physiological NaCl solution was used in the preparation of the epithelial cells as is usually done cytochrome *a*₃ concentration in the intestinal mitochondria obtained was very low (Cyt *a*₃: Cyt *cc* = 1:0.76). This indicates that 0.9% NaCl can extract some components from the intestinal cells and is not suitable for isolation of intestinal epithelial cells for studies on energy metabolism.

References

- DE JONGE H. R. and W. C. HULSMAN *Eu. J. Biochem.* 1973 32 356-364.
 HARRISON D. D. and H. L. WEBSTER *Exp. Cell Res.* 1969 55 257-260.
 KIMURA T., J. HAUBER and T. P. SINGER *J. Biol. Chem.* 1967 242 4987-4993.
 KING T. E. *Methods Enzymol.* 1967 10 322-331.
 WILSON D. F. *Arch. Biochem. Biophys.* 1967 121 757-768.

The effect of intracranial stimulation of the seventh cranial nerve (n. facialis-n. intermedius) on the blood flow of the eye and the tongue was studied in rabbits. The nerve was electrically stimulated with a stereotaxic technique: pulse intensity 6-14 V, duration 1 msec, and frequency 40-90 Hz. Regional blood flow in the eye and the tongue was measured with radioactively labelled microspheres.

Stimulation caused a $12 \pm 39\%$ ($n=18$) ($p<0.001$) increase in choroidal blood flow on the stimulated side as compared to the control side. Flow rates in the ciliary processes and the iris increased by $93 \pm 19\%$ ($n=18$) ($p<0.02$) and $11 \pm 5\%$ ($n=18$) ($p<0.001$) respectively. Lental blood flow also tended to increase during stimulation. The blood flow of the mucous membrane of the anterior part of the tongue increased by $210 \pm 64\%$ ($n=17$) ($p<0.01$) on the stimulated side. The blood flow of the corresponding muscle layer of the tongue also increased but with a great variability in the results. The duration of the vasodilation of the eye after a period of stimulation was 30-60 min while in the tongue it was about 10 min. Neither the increase in blood flow of the eye nor that of the tongue could adequately be abolished by cholinergic blockade with biperiden (5-10 mg/kg b.w.) or by ganglionic blockade with hexamethonium (50-100 mg/kg b.w.). Transection of the trigeminal nerve prior to stimulation of the facial nerve did not prevent the vasodilation.

The phenomenon of atropine-resistant vasodilation in the tongue as a result of cord stimulation is well-known and recent studies by Hellekant (1977) indicate that it is due mainly to activation of nerves originating in the CNS and not in the geniculate ganglion, that is to nerves which seem to be efferent. It is also known that close arterial injection of acetylcholine causes marked but short lasting vasodilation in the choroid in rabbits (Bell 1962).

The efferent autonomic nerves known so far in the intermediofacial nerve are cholinergic and have synapses in the pterygopalatine or submandibular ganglia. Such efferent nerves cannot explain the present results. The results rather indicate that the intracranial part of the intermediofacial nerve contains unconventional efferent or afferent fibres that on stimulation release a vasodilating substance in the eye and the tongue that is not acetylcholine.

References

- HELLEKANT T. G., *Acta Physiol. Scand.* 1977 99: 292-299.
BELL, A., *Acta Physiol. Scand.* 1962 46: 70-82.

D 10

Turnover of Noradrenaline, Dopamine and 5-Hydroxytryptamine in the CNS under the influence of 4-Aminopyridine

By NILS-ERIK ANDÉN and STEFAN LEANDER, Department of Pharmacology, Universities of Göteborg and Lund, Sweden

4-Aminopyridine (4-AP) enhances nerve impulse evoked release of both acetylcholine and noradrenaline from peripheral nerves (Leander et al. 1977, Lundh et al. 1977). 4-AP also

TABLE I Protein yield cytochrome concentrations succinate dehydrogenase activity and oxygen consumption using succinate as a substrate in mitochondria of intestinal epithelial cells. Values are mean values \pm S.E. from 1–14 determinations

Protein yield mg/100 g body wt	7.2 \pm 0.52
Cytochrome aa ₃ , nmol/mg protein	0.29 \pm 0.02
Cytochrome b nmol/mg protein	0.28 \pm 0.02
Cytochromes c+c nmol/mg protein	0.38 \pm 0.03
Cytochrome aa ₃ , Cytochromes c+c	1.13
Succinate dehydrogenase μ mol/min/mg protein	
Actual	0.22 \pm 0.02
Maximal	0.55 \pm 0.03
Oxygen consumption nmol/min/mg protein	
State 4	18.9 \pm 5.3
State 3	25.3 \pm 2.1
Respiratory control ratio State 3/State 4	1.4 \pm 0.06

In the present preliminary work conditions of the isolation of rat intestinal epithelial mitochondria and their general properties were determined. Intestinal epithelial cells of Sprague Dawley rats were separated principally according to Harrison and Webster (1969) but using 0.3 M mannitol—10 mM Tris—5 mM EDTA—3 mM MgCl solution pH 7.4. The cells were sedimented and intestinal mitochondria were isolated using the same medium. Mitochondrial cytochromes were determined in 1 M sucrose at liquid nitrogen temperature (Wilson 1967). Absorption coefficients of De Jonge and Hulsman (1973) were used for calculating the cytochrome concentrations. Both actual and maximum activity of succinate dehydrogenase were determined by the method of King (1967) by using substrate activation (Kimura *et al.* 1967). Mitochondrial oxygen consumption was determined using a Clark type oxygen electrode. The results are represented in Table I.

The present results also showed that if physiological NaCl solution was used in the preparation of the epithelial cells, as is usually done, cytochrome c concentration in the intestinal mitochondria obtained was very low (Cyt aa/Cyt cc = 1.076). This indicates that 0.9% NaCl can extract some components from the intestinal cells and is not suitable for isolation of intestinal epithelial cells for studies on energy metabolism.

References

- DE JONGE H. R. and W. C. HULSMAN *Eur J Biochem* 1973 32 356–364
 HARRISON D. D. and H. L. WEBSTER *Exp Cell Res* 1969 55 257–260
 KIMURA T. J. HAUBER and T. P. SINGER *J Biol Chem* 1967 242 4987–4993
 KING T. E. *Methods Enzymol* 1967 10 322–331
 WILSON D. F. *Arch Biochem Biophys* 1967 121 757–768.

D 9

Effect of Facial Nerve Stimulation on the Blood Flow of the Eye and Tongue

By JOHAN STJERNESCHANTZ and ANDERS BILL, *Institute of Physiology and Medical Biophysics, Biomedical Centre, University of Uppsala, Sweden*

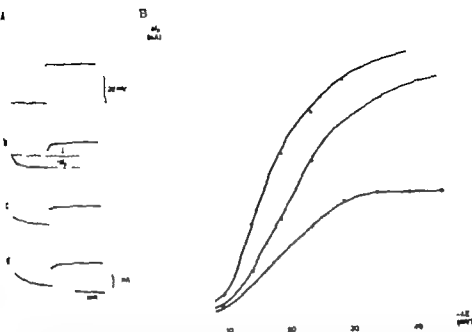


Fig. 1. A, recordings of membrane polarization (trace a) and membrane current in slowly (trace b) and in fast (traces c and d) adapting lobster stretch receptor neurone. Traces b and c were obtained in 5 mM, and trace d in 10 mM extracellular K. B, relationship between membrane hyperpolarisation ($-V_m$) and pump current decrease ($-\Delta I_p$, as defined in panel A) in a slowly adapting cell (triangles) and in fast adapting cells (circles) in 5 mM (open symbols), and in 10 mM (filled symbols) extracellular K.

In lobster stretch receptor neurones slow and reversible decreases in outward membrane current can be produced by hyperpolarising voltage clamp pulses (Fig. 1 A). In comparable conditions these decreases are always much larger in slowly than in fast adapting cells. In both cells they are diminished by all procedures which lead to a reduction in Na-K pump activity. Such procedures include cooling, treatment with ouabain or 2,4-dinitrophenol, and lowering the perineuronal K concentration. Conversely the voltage dependent current decreases are enhanced by pump stimulating procedures such as warming or increasing the extracellular K concentration (Fig. 1 B). For these reasons they are regarded as evidence of the Na-K pump and its pump current being voltage-dependent in hyperpolarising potential regions.

Possibly this voltage dependence is mediated by K whose concentration near the pump may vary with voltage dependent variations in transmembrane K leakage on condition that diffusion barrier existed between the perineuronal space and the surrounding fluid compartment. In such circumstances, polarisation induced reversal of the transmembrane K leakage might lower the perineuronal K concentration below normal levels and thus produce a reduction in Na-K pump activity.

produces CNS-stimulation (Jankowska et al 1977) by enhancing the release of various transmitters. Therefore it was of interest to investigate the effect of 4-AP on the turnover of the central monoamines noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT).

The experiments were done on intact or spinalized male Sprague Dawley rats. The amine concentrations in the brain and in the spinal cord were determined fluorometrically after cation exchange chromatography. 4-AP was given at a dose of 3 mg/kg i.p. which is the highest subconvulsive dose.

4-AP did not markedly influence the concentration of 5-HT or DA either alone or in combination with the synthesis inhibitors α -propylidopacetamide or α -methyltyrosine. 4-AP did not change the endogenous concentration of NA, but it significantly accelerated the α -methyltyrosine-induced disappearance of NA in the brain and in the intact spinal cord. This effect of 4-AP was not influenced by atropine indicating that it is not mediated via muscarinic acetylcholine receptors. The stimulatory effect of 4-AP on NA-utilization was however completely inhibited caudal to a section of the spinal cord indicating that this process depends on nerve impulses.

The α -adrenoreceptor stimulating agent clonidine almost completely inhibited the stimulatory effect of 4-AP on the NA turnover. Thus it is unlikely that 4-AP accelerates the turnover of NA by blocking α -adrenoreceptors. A subconvulsive dose of pentylenetetrazol producing about the same behavioural changes as 4-AP did not markedly effect the utilization of NA indicating that the effect of 4-AP is not caused by its convulsive activity. A possible explanation for the effect of 4-AP on the turnover of NA is an increased entry of calcium into nerve terminals causing increased transmitter release. Such a mechanism has already been suggested to explain the effects of 4-AP on acetylcholine release from motor nerve terminals (Lundh and Thesleff 1977). The absence of effect of 4-AP on the utilization of DA and 5-HT might be explained by a release mechanism less sensitive to calcium for these two transmitters (Chase et al 1969, Arbilla and Langer 1978).

References

- ARBILLA S and L. Z. LANGER *N. t. e.* 1978 271 559-561.
CHASE, T. N., R. I. KATZ and I. J. KOPIN *J. Neurochem.* 1969 16 607-615.
JANKOWSKA E., A. LUNDBERG, P. RUDOMIN and E. SYKOWA *Brain Res.* 1977 136 387-392.
LEANDER S., A. ARNER and B. JOHANSSON *Eu. J. Pharmacol.* 1977 46 351-361.
LUNDH H., S. LEANDER and S. THESLEFF *J. Neurol. Sci.* 1977 32 29-43.
LUNDH H. and S. THESLEFF *Eu. J. Pharmacol.* 1977 42 411-412.

D 11

Polarisation Dependence of the Na-K Pump in Lobster Stretch Receptor Neurotes

By C. G. Å. EDMAN and W. GRAMPP *Department of Physiology and Biophysics
University of Lund, Sweden*

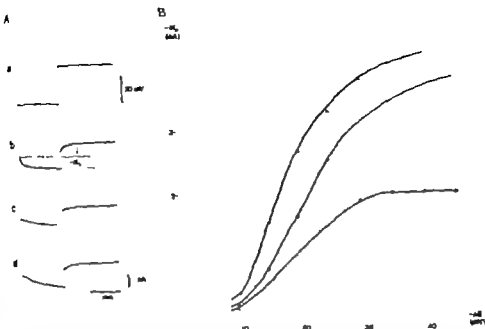


Fig. 1 A recordings of membrane polarization (trace a) and membrane current in a slowly (trace b) and in fast (traces c and d) adapting lobster stretch receptor neurons. Traces b and c were obtained in 5 mM, and trace d in 10 mM extracellular K. B relationship between membrane hyperpolarization ($-\Delta V_m$) and pump current decrease ($-\Delta I_{pump}$) as defined in panel A) in slowly adapting cell (triangles) and in fast adapting cell (circles) in 5 mM (open symbols), and in 10 mM (filled symbols) extracellular K.

In lobster stretch receptor neurones slow and reversible decreases in outward membrane current can be produced by hyperpolarising voltage 'ramp' pulses (Fig. 1 A). In comparable conditions these decreases are always much larger in slowly than in fast adapting cells. In both cells they are diminished by all procedures which lead to a reduction in Na-K pump activity. Such procedures include cooling, treatment with ouabain or ω ,4-dinitrophenol, and lowering the perineuronal K concentration. Conversely the voltage dependent current decreases are enhanced by pump stimulating procedures such as warming or increasing the extracellular K concentration (Fig. 1 B). For these reasons they are regarded as evidence of the Na-K pump and its pump current being voltage dependent in hyperpolarising potential regions.

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References

- ARBILLA S and S Z LANGER *Nature* 1978 271 559-561
 CHASE, T N, R I KATZ and I J KOPIN *J Neurochem* 1969 16 607-615
 JANKOWSKA E, A LUNDBERG, P RUDOMIN and E SYKOWA *Brain Res* 1977 136 387-392
 LEANDER S, A ARNER and B JOHANSSON *Eur J Pharmacol* 1977 46 351-361
 LUNDH H, S LEANDER and S THESLEFF *J Neurol Sci* 1977 32 29-43
 LUNDH H and S THESLEFF *Eur J Pharmacol* 1977 42 411-412

D 11

Polarisation Dependence of the Na-K Pump in Lobster Stretch Receptor Neurons
 By C G Å EDMAN and W GRAMPP *Department of Physiology and Biophysics*
University of Lund, Sweden

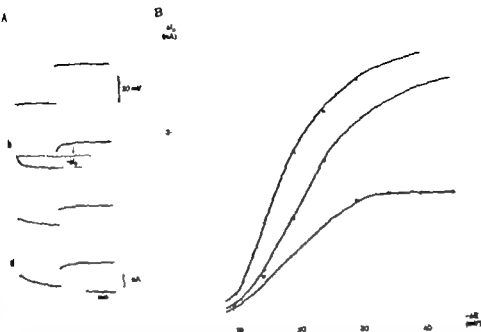


Fig. 1. A: recordings of membrane polarization (trace a) and membrane current in slowly (trace b) and in a fast (traces c and d) adapting lobster stretch receptor neurone. Traces b and c were obtained in 3 mM, and traces d in 10 mM extracellular K. B: relationship between membrane hyperpolarization ($-\Delta V_m$) and pump current decrease ($-\Delta I_p$, as defined in panel A) in a slowly adapting cell (triangles) and in fast adapting cell (circles) in 3 mM (open symbols), and in 10 mM (filled symbols) extracellular K.

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References

- ARBILLA E and S. Z. LANGER *Nature* 1978 271 559-561
 CHASE T N, R. I. KATZ and J. J. KOPPEL *J Neurochem* 1969 16 607-615
 JANKOWSKA E, A. LUNDBERG, P. RUDOMIN and E. SYKOWA *Brain Res* 1977 136 387-392
 LEANDER S, A. ARNER and B. JOHANSSON *Eur J Pharmacol* 1977 46 351-361
 LUNDH H, S. LEANDER and S. THESLEFF *J Neurol Sci* 1977 32 29-43
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D II

Polarisation Dependence of the Na-K Pump in Lobster Stretch Receptor Neurons

By C. G. Å. EDMAN and W. GRAMPP *Department of Physiology and Biophysics*
University of Lund, Sweden

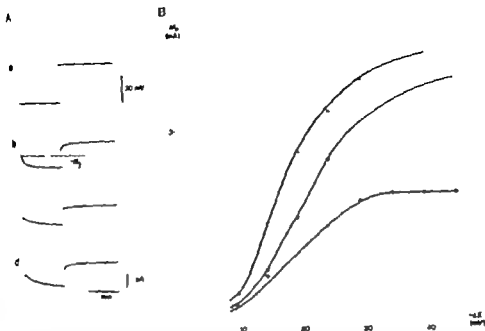


Fig. 1 A recordings of membrane polarization (traces a) and membrane current in a slowly (traces b) and in fast (traces c and d) adapting lobster stretch receptor neurons. Traces b and c were obtained in 5 mM and traces d in 10 mM extracellular K^+ . B relationship between membrane hyperpolarization ($- \Delta E_m$) and pump current decrease ($- \Delta I_{Na}$, as defined in panel A) in slowly adapting cell (triangles) and in a fast adapting cell (circles) in 5 mM (open symbols) and in 10 mM (filled symbols) extracellular K^+ .

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D 11

Polarisation Dependence of the Na-K Pump in Lobster Stretch Receptor Neurons

By C G Å EDMAN and W GRAMPP *Department of Physiology and Biophysics
 University of Lund, Sweden*

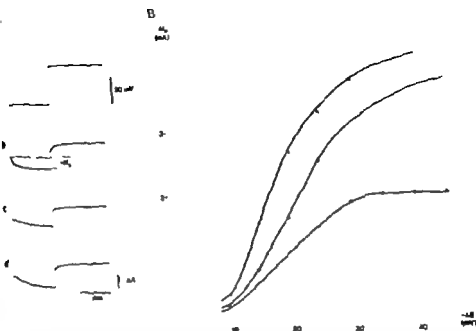


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- ARBILLA S and S Z. LANGER *Nature* 1978 271 359-361
 CHASE, T N, R I KATZ and I J KOPIN *J Neurochem* 1969 16 607-615
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D 11

Polarisation Dependence of the Na-K Pump in Lobster Stretch Receptor Neurons

By C. G. Å EDMAN and W. GRAMPP *Department of Physiology and Biophysics
 University of Lund, Sweden*

D 14

Uterine Secretomotor Innervation

By M. HAMMARSTRÖM and N. O. SJÖSTRAND *Department of Physiology Karolinska Institutet Stockholm Sweden*

Nerves to the endometrial linings have been noted in several morphological studies. No functional study seems to have been made. Here data indicating a cholinergic secretomotor service supply to the endometrium are presented. The estimation of uterine secretion was based on the fact that uterine mucus contains carbohydrates.

Free-dissected uterus from guinea-pigs treated with estrogen and progesterone for 10-14 days were divided through the cervix, each horn was everted and mounted on an electrode (Sjöstrand and Swedin, 1970) and put into an organ bath containing Tyrode. Some animals were deservated 10-12 days before experiment. Some experiments were performed with the pelvic or the hypogastric nerve attached to the organ. After a stabilization period of 80 min. in ordinary Tyrode the organ was exposed to five sampling periods of 10 min. in glucosefree Tyrode in order not to disturb the carbohydrate determination. Each sampling period was interluded by 20 min. of stabilization and nourishment.

The first third and fifth period was used to determine resting level secretion, during the second and fourth period the organ was exposed to nerve field stimulation (0.5 Hz 30 V 0.1 ms) to hypogastric or pelvic nerve stimulation (5 Hz 30 V 0.1 ms) and/or to drugs.

The carbohydrate amount of the collected fluid (25 ml) was analyzed according to Dische and Popper (1926) and calculated as glucose.

Field stimulation produced an increase by $71\% \pm 6.8$ in carbohydrate secretion. This increase was abolished by TTX (3×10^{-6} M) or scopolamine (1.3×10^{-6} M). Sotalol (1.8×10^{-6} M) and phentolamine (1.6×10^{-6} M) did not affect the answer. Pelvic nerve and coxisternum connexion deservation did not influence the increase in carbohydrate secretion due to field stimulation. However hypogastric deservation reduced the answer to one third and para-cervical ganglion deservation almost abolished the answer. Hypogastric nerve stimulation resulted in an increased secretion by $40\% \pm 3.2$ whilst pelvic nerve stimulation apparently not affect the carbohydrate secretion ($8.7\% \pm 8.7$). Carbacholine increased the answer with a sharp threshold at 5.5×10^{-8} M and maximum reached at 5.5×10^{-7} M.

Thus we suggest a cholinergic sympathetic secretomotor supply to the guinea-pig endometrium the hypogastric nerve being the main path and some fibers relaying in the para-cervical ganglia.

References

- DISCHE, Z. and H. POPPER, *Biol. Zeitsung* 1926 175 371-411
 SJÖSTRAND N. O. and G. SWEDIN, *Acta Physiol. Scand.* 1970 80 172-177

The fact that at a given external K concentration the polarisation dependent Na-K pump inhibition is smaller in the fast than in the slowly adapting cell corresponds with the observation of a lesser pump activity in the former cell. This in turn may be related to the possibility that because of a smaller transmembrane K leakage the perineuronal K concentration was lower in the fast than in the slowly adapting cell.

D 12

Tonic Contraction in Chemically Skinned Fibres from the Anterior Bypass Retractor of the Mollusc *Mytilus edulis* (ABRM)

By F. CORNELIUS *Biophysics Institute Aarhus University Denmark*

Dependent on the method of stimulation the living ABRM can produce two kinds of contractions. Repetitive electrical shocks cause a phasic contraction which is characterised by a high relaxation rate. Acetylcholine (ACh) induces a tonic contraction. In the presence of ACh the developed tension remains nearly constant and the muscle is active, judged from quick release experiments. A third state ('catch') appears when ACh is washed out. Relaxation is much slower than for phasic contraction and active state disappears progressively with time (Jewell 1959). Distinction between the two types of contractions and the catch state can thus be made by the time course of relaxation and the degree of active state.

In ABRM fibre bundle preparations treated with the nonionic detergent saponin (Endo et al. 1977) contraction is initiated when the fibres are exposed to activation solution containing a high free Ca^{2+} concentration ($p\text{Ca}=4.5$). The muscle can produce maximal tension over a period of several hours without decay of the active state. When a relaxation solution ($p\text{Ca} > 8$) is applied the muscle relaxes extremely slowly and the active state declines with time. The rate of relaxation can be greatly increased by applying serotonin or AMP. The chemically skinned fibres can thus be made to produce contractions with the same characteristics as for tonic contraction and catch state in the living ABRM.

The response of the preparation to varying Ca^{2+} concentration was found to be dependent on the free Mg^{2+} concentration. At very high Ca^{2+} concentrations ($p\text{Ca} < 4$) the force generating capacity of the muscle was depressed. This depressant effect could however be eliminated by raising the free Mg^{2+} concentration. At optimal Ca^{2+} concentration an increase in the Mg^{2+} concentration caused an increase in the rate of relaxation.

The results suggest that Mg^{2+} may play a role in the regulation of the catch state.

References

- ENDO M, T. KITAZAWA S, YAOI M, INO and Y. KAKUTE: *Excitation-Contraction Coupling in Smooth Muscle*. Eds R. Casteels et al. Elsevier/North-Holland Biomedical Press, 1977, 199-209.
JEWELL, B. R. *J. Physiol. (Lond.)* 1959, 149, 154-177.

transport kinetics is different in the three groups of animals. From a double reciprocal plot a K_t of 4 mM, 11 mM and 22 mM was calculated for the group with low—normal—and high cerebral glucose concentration respectively.

A model of the blood-brain barrier with one Michaelis-Menten membrane in the capillary wall was fitted to the data. A reasonable fit was obtained with small variations of K_t . A model constituted of two Michaelis-Menten membranes in series symbolizing the luminal and the contraluminal endothelial cell membrane gave an even better fit with constant K_t values. Thus the apparent increase of K_t with increasing cerebral glucose concentrations seems to reflect a methodological problem rather than a real change of the transport mechanism. This conclusion has a bearing on many experiments on glucose transport in the brain (Lund-Aandersen 1979).

References

- BEZ, A. L. D., B. GILBERT and L. R. DIERCKX, *Biochim. Biophys. Acta* 1975 401 416-428.
 CRONE, C., *J. Physiol. (Lond.)* 1965 181 103-113.
 LUND-ANDERSEN, H. *Physiol. Rev.* 1979 59 In press.
 OLSEN, W. H. *Amer. J. Physiol.* 1971 221 1629-1639.

D 16

Contracture-Potentiating Effects of the SH Blocking Para-Mercuribenzoates

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The mercuribenzoate sulfhydryl-blocking agents pCMB, pCINBS and pOHMB (100 μ M) cause no contractures in the rat diaphragm preparation by themselves, but potentiate the contracture response caused by other agents. These effects are described and their dependency on the myotonia caused by the SH-blocking agents (Røed 1976) and the significance of SH binding for the effects are investigated.

The biphasic caffeine (10 mM) contracture was increased in amplitude upon pOGBM (100 μ M) pretreatment. The first phase increased from 12.4 ± 3.5 per cent to 42.8 ± 14.5 per cent of the maximal twitch amplitude prior to drug addition. This maximum tension appeared after 7-9 min in the pOHMB-treated preparation, while in the control preparation it appeared after 25-30 min.

The phasic depolarization contracture caused by KCl (100 mM) was increased in amplitude from 70.7 ± 15.5 per cent to 125.2 ± 14.8 per cent after prior exposure to pOHMB (100 μ M). Moreover, the time of maximal amplitude was shortened from about 20 sec to 5-6 sec by pretreatment with pOHMB.

Some of the membrane-stabilizing drugs with local anaesthetic activity like propranolol and dibucaine caused a biphasic contracture in the 1 mM concentration. In the control preparation, the contracture was preceded by a delay of 2-3 min which was completely obliterated by pOHMB pretreatment. The amplitude of the first phase of the

Glucose Transport Across the Blood-Brain Barrier: On the Apparent Effect of the Cerebral Glucose Concentration upon the Transport Affinity Constant

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The transport of D-glucose from blood to brain across the blood-brain barrier exhibits Michaelis-Menten saturation kinetics (Crone 1965). It has been shown by Betz et al. (1975) that the transport affinity constant K_t apparently increases from 8 to 37 mM when the cerebral glucose concentration increases from 2 to 12 mM. However, in their type of non-steady state experiments the glucose concentration at the carrier sites on the luminal and on the contraluminal endothelial cell membrane might be underestimated when glucose concentration in the bolus was lower than that in the brain because glucose passes out of the brain through the endothelial cells into the blood. This may explain the apparent increase of K_t with increasing cerebral glucose concentrations.

This hypothesis was tested by fitting models of the blood-brain barrier transport to kinetic data obtained on rats with a cerebral glucose concentration which was low, normal and high. The data were obtained by aid of the non-steady state single injection technique developed by Oldendorf (1971). The results are given in Fig. 1. It appears that the

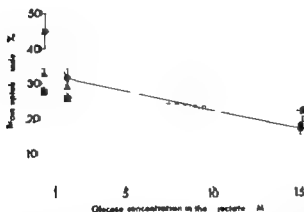


Fig. 1. Transport of the non-metabolizable glucose analogue ^{14}C 3-O-methylglucose across the blood-brain barrier relative to the transport of $^3\text{H}_2\text{O}$ (brain uptake index BUI) after injection of an isotope-containing bolus in the common carotid artery (Oldendorf 1971). Decapitation was performed 5 sec after injection. The inhibitory effect of increasing glucose concentrations (0.17 mM, 1.5 mM and 15 mM) in the injectate on the transport of labelled 3-O-methylglucose was studied on three groups of rats which prior to injection were in steady state at different plasma and brain glucose concentrations: (●-●) indicates plasma glucose of 1 mM and brain glucose of about 6 mM, (▲-▲) indicates plasma glucose of 7 mM and brain glucose of about 10 mM and (■-■) indicates plasma glucose of 35 mM and brain glucose of about 10 mM. Each point is the mean of 3-6 experiments with S.E.M. indicated by vertical bars.

permeability was negligible indicating that there was no significant contribution of choroidal blood to the plexus.

In anaesthetized pigs the following a-v-differences in mMol/l were observed for the retina and choroid respectively (mean \pm SE and number of experiments): Glucose 0.49 ± 0.06 (13) 0.14 ± 0.06 (10) Oxygen 2.50 ± 0.24 (12) 0.15 ± 0.03 (11) Lactate— 0.25 ± 0.10 (8)— 0.29 ± 0.11 (8) All differences were significant at the $p < 0.05$ level or less. Studies with the labelled microsphere technique provided data for retinal and choroidal blood flow in anaesthetized pigs 64 and 1732 mg/min respectively. Then if the metabolism of the choroid is neglected and the admixture of blood from the anterior uvea is assumed to cause no major change in venous O_2 , glucose and lactate concentrations it can be estimated that the contribution to retinal metabolism from the retinal vessels is 161 and 21 μ Mol/min and from the choroid 260 and 242 μ Mol/min of oxygen and glucose respectively. The oxygen provided can only account for complete oxidation of 26% of the glucose. A major part of the glucose thus seems to be metabolized to lactate. The values for glucose and oxygen consumption and the lactate production found in the present study agree well with the results obtained in *in vitro* studies on rats (Reading and Sorsby 1962).

References

- BLAND, H. W. and A. SORSBY *Vision Research* 1972, 2, 315-325
 TONQUEST, P. 9th Europ. Conf. Microcirculation, Antwerp 1976. *Bull. anat.* N 16, 51-55 (Karger, Basel 1977).

D 18

Sympathetic Effects on Cerebral Circulation in Acute Stress

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The blood vessels of the brain have a rather rich supply of sympathetic nerves. The physiological role of these nerves has been debated upon for a long time (Laason 1974). During normotension electrical stimulation of the cervical sympathetic chain has practically no effect on cerebral blood flow as determined with labelled microspheres (Alm and Bill 1973). In acute hypertension in anaesthetized animals sympathetic stimulation has been shown to have significant effects (Bill and Linder 1976). Stimulation helps to prevent forced vasodilatation, overperfusion with blood and breakdown of the blood-brain barrier.

The purpose of these experiments was to study the physiological importance of the sympathetics in acute stress. The experiments were performed in awake SHR rats. The animals were cannulated and unilaterally sympathectomized the day before the experiment. The acute stress and blood pressure rise was caused by forcing the animals to swim in cold water. The blood-brain barrier was studied using 125-Iodinated albumin. As a microscopic tracer Evans blue was used. A rise in MABP of 40-105 cm H_2O was induced. The pressure rise tended to cause a break down of the blood-brain and blood-aqueous barriers, effects that seemed to be more marked on the sympathectomized side.

propranolol contracture was 40.8 ± 8.4 per cent in the control preparation and 48.4 ± 15.7 per cent after pOHMB pretreatment. This increase was not significant.

The membrane stabilizer diphenylhydantoin which lacks local anaesthetic activity caused a monophasic contracture with a maximal amplitude of 10.0 ± 3.2 per cent within 2 min in the pOHMB-treated preparation in 1 mM concentration. This contracture was not observed in the control preparation.

Myotonia appeared upon pretreatment with pOHMB. However the contracture potentiating effect was independent upon the presence of myotonia. Selective block of the myotonic response with a local anaesthetic did not reduce the contracture-potentiating ability and experiments on the slow twitch rat soleus muscle showed high contracture-potentiating ability combined with no myotonic response.

All the contracture-potentiating effects of pOHMB were dependent on the SH-binding properties of pOHMB because these effects were not observed when the preparations were pretreated with dithiothreitol (100 μ M) which keeps the SH groups in the reduced state (Cleland 1964).

On the other hand contracture potentiation was not a general result of SH inhibition. It was not observed after pretreatment with N-ethylmaleimide another SH-blocking agent.

In conclusion it is suggested that the mercuribenzoates interact with SH groups and thereby indirectly increase the availability of the Ca compartments being released by the contracture producing agents.

References

- CLELAND W W *Biochemistry* 1964 3 480-482
ROED A *Acta physiol scand* 1976 Suppl 440 118

D 17

Retinal and Choroidal Contribution to Retinal Nutrition

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The retina is supplied from two sources: the retinal capillaries and the choroidal capillaries. The retinal metabolism under *in vivo* conditions can be studied by determining α -v-differences for substrates and metabolites: venous blood being sampled both from the choroid and the retina. Choroidal blood with a moderate admixture of blood from the anterior uvea can be obtained by cannulating a vortex vein. Retinal venous blood is difficult to obtain due to the inaccessibility of the retinal veins. A technique was developed in pigs that permits cannulation of a venous plexus around the optic nerve. By aid of silicone rubber casts this plexus was shown to drain retinal blood. Previous studies with the single injection technique (Törnquist 1977) have shown a considerable permeability to sodium in the choroid. This technique applied to the retina showed that the sodium

vascular disturbance the perfusion of the nerve head being reduced to levels causing undernutrition of the nervous tissue. The arrangement of the vessels supplying the distal part of the nerve is complex: branches from the central retinal artery and short posterior ciliary arteries supplying poorly defined regions. It has been suggested that elevation of the intraocular pressure causes a redistribution of the nerve head blood flow to extraocular spaces and also that autoregulation of the optic nerve blood flow might be poor compared to that in the retina.

In monkeys the arrangement of the vascular supply to the optic nerve is similar to that in man and model glaucomas can be produced. We have now used unlabelled 8-10 μ m microspheres to study the effects of acute and subacute elevations of the intraocular pressure on the blood flow in the different regions of the peripheral part of the optic nerve. The regions investigated were the prelaminar part of the nerve, the lamellar part and the first, second and third millimeters of the nerve behind the lamina. The central retina was also investigated. One eye had its normal eye pressure and the other had its pressure stabilized at a higher level to reduce the perfusion pressure defined as the mean arterial pressure minus the intraocular pressure. Unlabelled microspheres were injected into the left heart ventricle. The eyes were frozen and sectioned and the number of spheres in the different regions counted. The results indicate that there is efficient autoregulation of the blood flow in the retina and optic nerve head within a wide range of perfusion pressures. Lamellar flow is not stopped at pressures stopping flow in the eye and close lamellar flow behind the lamina is increased at high intraocular pressures most probably due to diffusion of metabolites from the prelaminar region through the lamina. After a period with high intraocular pressure reduction in eye pressure results in reactive hyperemia in the retina, and in the prelaminar and lamellar parts of the nerve. The results do not offer any simple explanation for the development of optic nerve damage in glaucoma.

D 21

The Time Course of Adaptation in the Retinular Cells of the Fly

By J. MORINO, M. JARVILEHTO and K. MORINO *Department of Physiology, University of Oulu, Finland*

The sensitivity of photoreceptor cells is controlled by photomechanical, photochemical and neural mechanisms. The amount of energy required to activate the photochemical mechanisms is relatively high and photomechanical reactions are known to be slow. At low levels of light energy the neural mechanism is responsible for fast changes of light sensitivity in photoreceptor cells (for review see Goldsmith (1975)). This mechanism is believed to affect the chain of chemical events leading from the absorption of light quanta to the electrical response of the cell. Another possibility for sensitivity control is an inhibitory feedback. There is evidence of both in vertebrate and invertebrate eyes.

In the case of inhibitory feedback alone as an adapting mechanism, the onset of sensitivity reduction would be delayed. On the other hand a pure membrane mechanism could lead to the reduction of sensitivity already during the latency time of the photoreponse.

References

- ALM A and A BILL *Acta physiol scand* 1973 88 84-94
 BILL, A and J LINDER, *Acta physiol scand* 1976 96 114-121
 LASSEN N A *Circulat Res* 1974 34 749-760

D 19

A Method to Determine the Rate of Aqueous Humor Formation Some Preliminary Results

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The aqueous humor produced by the ciliary processes flows from the posterior chamber into the anterior chamber and is drained in the iridocorneal angle in such a way that it cannot be collected quantitatively. Indirect studies in the rabbit have indicated that changes in the pH of the blood produced by infusion of sodium bicarbonate or hydrochloric acid have marked effects on aqueous humor formation. Alkalosis stimulated formation, acidosis inhibited (Krupin et al 1977).

We have developed an isotope dilution procedure for direct studies on aqueous humor production in monkeys on-line analysis of dilution data making it possible to determine aqueous humor formation rate every few minutes at normal intraocular pressures. Push-pull-coupled syringes are used to perfuse the anterior chamber with a solution of radioactive albumin at a rate of 1.6 $\mu\text{l}/\text{min}$. An external circuit and a circulation pump are used to mix the fluid in the anterior chamber and pump fluid through a gamma-detector. Counting rates are determined and treated by a minicomputer and flow rates calculated every second minute. The flow rates calculated 1-2 $\mu\text{l}/\text{min}$ are fairly steady over hours and agree well with known data for monkeys. Infusion of sodium bicarbonate and hydrochloric acid have smaller effects in monkeys than in rabbits. Indomethacin 2-20 mg/kg b.w. has no effect on the normal flow rate but seems to prevent an irritative response otherwise sometimes appearing after 4-5 hours of perfusion.

Reference

- KRUPIN T, CH J, OESTRICH J, BASS S, PODOS and B BECKER *Invest Ophthalmol & Visual Science* 1977 16/11 997-1001

D 20

Optic Nerve Blood Flow Measured with Microspheres. Effects of Ocular Hypertension

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In glaucoma there is usually a high pressure in the eye and it has been assumed that the degeneration of the optic nerve that is characteristic of the disease is a consequence of a

Table 1 Effect of insulin and adrenaline of ^{22}Na -efflux, ^{42}K -influx and E_{K} in rat soleus muscle. For details see Clausen & Flatman, 1977. All results given \pm S.E. with the number of observations in parentheses. E_{K} the number of fibres penetrated in the number of muscles investigated.

	^{22}Na -efflux (fraction of ^{22}Na lost per sec)	p	^{42}K -influx ($\mu\text{moles/g/}$ min)	p	ΔE_{K} (mV) (increase in E_{K} in relation To untreated control muscles)	p
control	0.045 ± 0.003 (3)		0.6 ± 0.02 (8)			
insulin		<0.005		<0.005		
insulin + adrenaline	0.081 ± 0.005 (3)		0.74 ± 0.02 (8)		3.5 ± 0.4 (224/6)	<0.001
insulin + adrenaline	0.106 ± 0.003 (3)	>0.10	0.80 ± 0.03 (8)	<0.01	7.7 ± 0.3 (740/6)	<0.001
insulin + adrenaline + albutamol	0.110 ± 0.003 (3)		0.90 ± 0.02 (8)		6.6 ± 0.4 (230/6)	<0.001

<0.05

both hormones indicating it is related to a stimulation of active Na-K transport. The effect of adrenaline and insulin were partially additive on Na-K transport whether insulin was added before, after or simultaneously with (Table 1) adrenaline. However the hyperpolarization produced by insulin and adrenaline combined (insulin added before adrenaline) was no larger than that produced by adrenaline alone. Indeed the hyperpolarization produced by adrenaline is reduced by the later addition of insulin.

Also *in vivo* (pentobarbitone anaesthetized ~ 50 g rats) insulin and adrenaline (or albutamol) individually increased E_{K} in combination their actions were additive.

References

- CLAUSEN, T and J A FLATMAN. *J Physiol* 1977 270 383-414.
CLAUSEN, T and P M KORY. *J Physiol* 1977 265 19-42.
ZIEGLER, K L. *Handbook of Physiology* section 7 Endocrinology. Williams and Wilkins Baltimore 1972, pp 347-368.

D 23

Role of Physical Fitness in Lipolytic Activities of Human Postheparin plasma

By P. PELTONEN, J. MARNIEMI, I. VUORI and E. HIETANEN. *The Rehabilitation Research Centre of the Social Insurance Institution, Turku and Department of Physiology, University of Kuopio, Finland.*

The triglyceride lipase activities released into the blood circulation by heparin injection have been studied widely in certain disordered states of fat metabolism. The effect of physical exercise on human lipolytic enzymes is still obscure. In laboratory animals endurance training has increased significantly lipoprotein lipase activity of the skeletal

To reveal which mechanism is responsible for light adaptation we measured the time course of adaptation by using an adapting flash and a test flash both with a duration of 10 μ s and an estimated maximum quantum content of 10^{12} . The change of sensitivity was measured between 0 and 300 ms after the adapting flash. The latencies of the photoresponses were between 3 and 7 ms and the duration of the response up to 70 ms. The amplitudes (maximally up to 40 mV) of the responses to the test flashes were converted to effective intensities. The relationships of these and the effective intensity of a nonadapted control response were used to describe the sensitivity of the cell. The effect of nonlinear summation of the responses to adapting and test flashes on sensitivity was eliminated by using the $V \log I$ functions of the cells. The sensitivity of the receptor cells began to decrease immediately after the adapting flash even before the electrical potential response was produced. This indicates that sensitivity is controlled at the beginning of the chain leading to the photoresponse and the mechanism does not require a change of membrane potential. The decrease of sensitivity continued to at least 15 ms and turned to dark recovery between 25 and 30 ms. The recovery was complete at approximately 300 ms in our experimental conditions. The nature of the mechanism is probably a release of an inhibitory substance (Ca^{2+}) at the beginning of the chain leading to the photoresponse. Light adaptation can further be reinforced by inhibitory feedback which can control the sensitivity only after the photoresponse is produced.

Reference

- GOLDSMITH T. H. Photoreceptor processes. Some problems and perspectives. *J. exp. Zool.* 1975 194: 89-102.

D 22

Combined Effect of Adrenaline and Insulin on Active Electrogenic Na-K Transport in Rat Soleus Muscle

By J. A. FLATMAN and T. CLAUSEN *Institute of Physiology, University of Aarhus, Denmark*

In the isolated soleus muscle of the rat, adrenaline has been shown to stimulate the active electrogenic Na-K transport (Clausen & Flatman 1977). Insulin likewise stimulates active Na-K transport (Clausen & Kohn 1977) and in rat EDL muscle the membrane potential (E_m) is increased (Zierler 1977). We have now compared the actions of these agents alone and combined in both "in vitro" and "in vivo" experiments.

Na-K transport and E_m were measured in soleus muscles from 60-70 g fed rats. In order to obtain more stable recordings of E_m , several series of measurements were performed on rats weighing \approx 250 g.

Supramaximal concentrations of either insulin or adrenaline stimulate ^{22}Na efflux, ^{42}K influx and increase E_m (Table I). The effects of insulin were somewhat less pronounced and slower in onset than those of adrenaline. Ouabain blocked the hyperpolarizing effect of

D 24

Bradycardia in Endurance Trained Men Due to Non-Autonomic Factors

By STEVEN LEWIS, PRAM GAD, EVA NYLANDER and NILS-HOLGER ARESKÖG. *August Krogh Institute University of Copenhagen Denmark and Dept of Clinical Physiology Regionshuset Linköping University Sweden*

The relative bradycardia of endurance training in man is to some extent attributable to changes in the autonomic nervous system (Ekbloom et al. 1973). However a lower resting heart rate (HR) after combined vagal and beta-adrenergic blockade has been found in endurance trained rats than in untrained rats (Hughson et al. 1976). In addition factors which establish the intrinsic HR in these animals seem to limit maximum exercise HR (Guthrie et al., 1976).

In the present study 8 healthy sedentary men (SED) (age 76 ± 4 yr; mean \pm SD) and 8 long distance competition bicyclists (ATH) (age $21 \pm$ yr) were compared at rest and during exercise on a bicycle ergometer at workloads corresponding to approximately 50, 75 and 100% of maximal oxygen uptake ($VO_{2\max}$). ATH had trained 400-700 km/week for 5-11 years prior to the study. Testing was performed under normal conditions and after combined vagal blockade with atropine (0.04 mg/kg b.w.) and beta-adrenergic blockade with propranolol (0.25 mg/kg b.w.) or metoprolol (0.5 mg/kg b.w.) injected intravenously. In nearly all comparisons very similar results were obtained after using metoprolol or propranolol. The exercise tests were carried out in ascending order of intensity and an additional dose of 0.05 mg/kg propranolol or 0.1 mg/kg metoprolol was injected prior to the 75% max. load.

$VO_{2\max}$ was 45 ± 6 (SED) and 76 ± 5 (ATH) ml/kg min after blockade (AB). $VO_{2\max}$ was reduced by approximately 70% in ATH and 10% in SED. Maximal HR was approximately 90 beats/min lower in both groups after AB. At rest and during exercise at 50, 75 and 100% $VO_{2\max}$ under normal conditions and after AB HR was lower in ATH than in SED ($p < 0.05$). The mean HR difference between SED and ATH was very similar in blockade (approximately 13 beats/min) at rest and at all levels of exercise under normal conditions and after AB.

These data indicate that prolonged intense endurance training may result in non-autonomic changes which reduce HR at rest and during exercise at any given level of relative cardiovascular stress. One non-autonomic factor which may play a role in the bradycardia is cardiac enlargement. Heart volume estimated from roentgenograms at rest was 420 ± 45 and 538 ± 78 ml/BSA in SED and ATH respectively ($p < 0.05$) and when the data from the two groups were combined ($N=16$) heart volume and resting HR were inversely related ($r = -0.64$).

References

- ELKROM, B. A., KILBOM, A. and J. SOLTYSIAK, *Scand J Clin Lab Invest* 1973 32 251-256.
 HUGHSON, R. L., J. D. FITZGERALD, J. R. BUTTON and H. L. JONES, *Int Congr Phys Act Sci* (Carter City 1976).



Fig. 1 Individual values of postheparin plasma hepatic/total lipolytic activity ratios in male non-obese trained (●) and nontrained control (○) subjects. The means are indicated by horizontal lines. The training consisted of skiing, running, swimming or other heavy exercise being repeated regularly several times a week. The significance of the difference between the means of the groups (calculated by Student's *t*-test) is: $P < 0.005$

muscle (Borensztajn et al 1975) but the effect of short term exercise in man was negligible (Marniemi et al 1978)

For the determination of the lipolytic activity the radiochemical method with ^3H -triolein as substrate as described by Huttunen et al (1975) was modified. The differentiation between hepatic and extra hepatic (lipoprotein) lipases was carried out utilizing the inhibition of extra-hepatic enzyme by 1.0 mol/l NaCl without any specific antiserum. The activity of hepatic lipase at this salt concentration is considerably higher than at 0.1 mol/l NaCl (Huttunen et al 1975) thus causing the hepatic enzyme/total activity ratio to be higher than one in some cases. Heparin (50 IU/kg) was administered intravenously into test subjects 10 min before the plasma sample was taken.

A positive correlation was found between the relative weight of the subjects and lipolytic activity. On the other hand a negative correlation was obtained between the serum triglyceride concentration and enzyme activity. The hepatic/total lipolytic activity ratio in postheparin plasma appeared to be decreased in actively training subjects when compared to non training controls (fig. 1). This difference is partly due to the decrease in the hepatic activity observed and partly perhaps due to increase in muscle lipoprotein lipase. The final clarification of this phenomenon in man awaits for the analysis of tissue samples and the plasma enzyme determination with the specific immunochemical method. These studies are in our next program.

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References

- BORENSZTAJN J, M S RONE, S P BABIRAK, J A MCGARRAND, L B OSCAL *Amer J Physiol* 1975, 229, 394-397
 HUTTUNEN, J K, C EHNHOLM, P K J KINNUNEN and E A NIKKILA *Clin chim Acta* 1975, 63, 335-347
 MARNIEMI J, P PELTONEN, I VUORI and E. HIETANEN *Acta physiol scand* 1978, 102, 38A-40A

D 26

The Effect of Fasting on the Response to Exercise of Catecholamines in Plasma

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Fasting profoundly changes the endocrine and metabolic state of the organism. We have now for the first time studied the sympathoadrenomedullary response to exercise after prolonged fasting.

6 healthy men (25 ± 2 yr (mean \pm SE); V_{O_2} max. 55 ± 1 ml/kg min) had blood drawn from the subclavian vein at rest and at the end of two successive treadmill runs lasting 10 and 5 min and requiring 50 and 100% of V_{O_2} max respectively. This was done after an overnight fast (C-expts.) and furthermore three times separated by 30 min rest intervals (D and F-expts.) after 39 h fasting. During the submaximal (0.75 g/min) and maximal (1.16 g/min) exercise in E-expts. a 30% (g/100 ml) glucose solution was infused.

Fasting increased heart rate at rest, submaximal and maximal exercise (88 ± 2 , $163 \pm 12 \pm 2$ beats/min (D-expts.) vs. 77 ± 4 , 151 ± 5 , 187 ± 3 (C-expts.)). Also noradrenaline values were significantly higher in D- than in C-expts. (0.65 ± 0.09 , 1.52 ± 0.17 , 6.64 ± 0.75 ng/ml vs. 0.30 ± 0.06 , 0.87 ± 0.10 , 3.41 ± 0.36) and adrenaline were higher during work (0.13 ± 0.02 , 1.43 ± 0.48 ng/ml (D-expts.) vs. 0.12 ± 0.01 , 0.42 ± 0.10 (C-expts.)). FFA, glycerol and hematocrit (43.9 ± 1.1 , 47.4 ± 0.8 , $48.0 \pm 0.8\%$ (D-expts.) vs. 43.5 ± 0.7 , 41.1 ± 0.7 , 47.1 ± 0.7 (C-expts.)) were higher and plasma glucose concentrations lower ($75.4 \pm 4.74 \pm 4.78 \pm 3$ mg/100 ml (D-expts.) vs. 94 ± 2 , 91 ± 3 , 94 ± 3 (C-expts.)) after fasting. During glucose infusion hematocrit values were similar to values in C-expts. and plasma glucose concentrations were higher (157 ± 10 , 191 ± 14). At the high workload heart rate (163 ± 1) and noradrenaline (5.25 ± 0.72) were significantly lower in E- than in D-expts. but adrenaline was at this as well as at the low workload still significantly higher than in C-expts. Also in the final treadmill run after fasting (F-expts.) the glucose concentrations were higher and hematocrit lower than in D-expts. but noradrenaline and heart rate were higher. Adrenaline concentrations were identical at similar work loads in the three exercise periods after fasting.

In conclusion, fasting markedly enhances the sympathoadrenomedullary response to exercise. The present study indicates that this enhancement is not explainable by changes in plasma glucose concentration or in plasma volume.

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Effect of Adrenal Demedullation on Hepatic Glycogen Depletion and Circulating Catecholamines in Exercising Rats

By E. A. RICHTER, H. GALBO and N. J. CHRISTENSEN *Institut of Medical Physiology B University of Copenhagen and Department of Internal Medicine and Endocrinology Herlev Hospital Copenhagen*

The Effect of Training in Relation to Chronological Age and Developmental Stages in Children 9 to 17 Years of Age

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The increase in dynamic endurance with training has previously been shown to be markedly greater in children aged 12 to 13 years than in both younger and older children (Ikai 1969). This suggests an increased sensitivity to training during puberty.

In order to test this hypothesis the above mentioned training experiment was reproduced using 240 children between the age of 9 and 17 years of age. The increase in dynamic endurance in the right arm flexors was measured and related to the chronological age and the following three developmental stages. The biological age (Greulich and Pyle 1959) and the pubertal growth spurt (Helm et al. 1971) were predicted from radiographs of the hand and wrist. The dental age (Demirjian et al. 1973) was obtained from a clinical investigation and radiographs of the teeth.

The results in relation to chronological age are not in agreement with the results of Ikai. We found that the relative mean increase in the dynamic endurance was 35% for the 13.5 years old boys and 29% for the 17.5 years old girls whereas in all other age groups it was found to be more than 50% for both sexes.

When the increase was compared with the different development stages a similar reduced increase was seen for the boys at the dental age of 13.5 years and for the girls at the dental age of 15.5 years. A reduced increase was also found at the biological age of 10.5 years for boys while no differences in increase was found for the girls. Finally when related to the predicted pubertal growth spurt the reduced increase was seen in the group of boys predicted to be half a year from the onset of the pubertal growth spurt. For the girls it was observed in the groups predicted to be in the middle of this growth spurt and half a year older.

Thus our results indicate that the increase in dynamic endurance with training is rather small at a certain chronological age and that this seems to be related to the period connected with the pubertal growth spurt. The separation of the results in means calculated for each of the two sexes and the earlier onset of the pubertal growth spurt seen in girls can to some extent explain the difference between these results and those previously reported.

References

- DEMIRJIAN A. H. GOLDSTEIN and J. M. TANNER *Human Biology* 1973 45 211-227
 GREULICH W. W. and S. I. PYLE, *Atlas* 1959
 HELM E. S. STERSBCK NIELSEN V. SKJELLER and A. BJØRK *Danish Dental Journal* 1971 75 1223-1234
 IKAI M. *F.I.E.P.* 1969 3/4 19-27

D 28

Sympathetic Atropine Sensitive Effects on Gastric Motility

D DELGRO and B. LISANDER, *Department of Physiology University of Göteborg
Göteborg, Sweden*

Efferent splanchnic nerve stimulation can decrease gastric tone by an activation of the adrenergic fibres. At low stimulation frequencies however atropine sensitive augmentatory responses may occur (Thomas and Baldwin 1968) and it was considered of interest to further analyse these less studied excitatory effects.

Experiments were performed on chloralosed adrenalectomized cats paralysed with gallamine and artificially ventilated. Gastric motility was recorded by a balloon, introduced via the oesophagus.

Efferent stimulation of the cut greater splanchnic nerves could either increase or decrease gastric tone. The excitatory responses occurred at higher stimulation intensities than the inhibitory ones but were as a rule observed at lower stimulation frequencies only (1-4 Hz). They were not prevented by bilateral cervical vagotomy, hexamethonium or guanethidine (both at 5 mg/kg) but could be completely abolished by atropine (0.1-0.5 mg/kg). Guanethidine and/or hexamethonium reversed inhibitory responses to excitatory ones and the latter then reached an optimum at 6-8 Hz or above.

Similar excitatory responses with the same pharmacological characteristics could be elicited when the thoracic sympathetic trunk was stimulated up to the level of the 7th thoracic vertebra and also from the centrally cut dorsal root at Th 10 in agreement with findings by Semba and Hiraoka (1957).

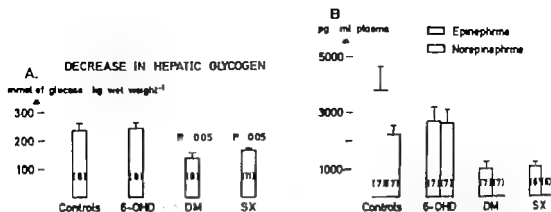
Efferent vagal stimulation in the neck did not evoke any action potentials in the greater splanchnic nerve indicating that the studied excitatory effects were not elicited by vagal fibres, running in sympathetic nerves.

Heating of a nerve can activate the thinnest afferents of the delta group and C-class (von Euler 1947). Heating of the greater splanchnic nerve caused an increase in gastric motility which could not be prevented by hexamethonium or guanethidine nor by cutting the nerve centrally. Atropine however completely abolished these responses.

The evidence thus suggests that the excitatory gastric responses to efferent splanchnic nerve stimulation are due to antidromic activation of afferent fibres. The functional significance of these effects remains obscure but it is possible that afferent fibres mediate excitatory axonal reflexes within the abdominal cavity.

References

- WELLER, C. *Acrophysiol. cond.* 1947 14 Suppl. 45 1-75.
SEMBA, T. and T. HIRAOKA *Jap. J. Physiol.* 1957 7 64-71.
THOMAS, J. E. and M. V. BALDWIN *Handbook of Physiology* Sect. 6 Vol. IV 1968 1937-1968.



We have reported (Galbo et al 1978) that in adrenalectomized rats injected with 6-hydroxydopamine (selectively destroying sympathetic nerve endings) hepatic glycogen depletion during exercise is smaller than in control rats. Now we have elucidated if this effect of sympathectomy can be ascribed selectively to the lack of either the adrenal medulla or of the peripheral sympathetic nerve endings.

Four groups of rats which 3 weeks previously had been either adrenalectomized (DM) injected with 6-hydroxydopamine (6-OHD) adrenalectomized and injected with 6-hydroxydopamine (SX) or sham operated and sham injected controls (C) were used. During ether anesthesia a small biopsy of the liver was removed and 15 min afterwards the rats were forced to swim with a tail weight for 100 min. Then blood was drawn and a liver sample was collected.

In DM and SX rats the declines in hepatic glycogen concentrations (Fig. 1 A) and the increases in blood glucose (Richter et al 1978) were significantly smaller than in 6-OHD and C rats. Furthermore in DM and SX-rats epinephrine in plasma was undetectable and the concentrations of norepinephrine were markedly below concentrations in C-rats (Fig. 1 B). 6-OHD did not significantly decrease the concentrations of norepinephrine and epinephrine but halved the norepinephrine/epinephrine ratio (2.38 ± 0.47 ($\bar{X} \pm SE$) in controls and 1.07 ± 0.09 in 6-OHD ($P < 0.05$)).

It is concluded that in the exercising rat 1) Circulating catecholamines enhance hepatic glycogen breakdown directly or indirectly 2) In contrast to the findings in man (Vendralu 1960) the major part of plasma norepinephrine is of adrenomedullary origin 3) Plasma epinephrine is entirely of adrenomedullary origin.

References

- GALBO H. E. A. RICHTER N. J. CHRISTENSEN and J. J. HOLST *Acta Physiol Scand* In press
 RICHTER E. A. B. SONNE J. J. HOLST and H. GALBO *Acta Physiol Scand* 1978 102: 13
 VENDRALU A. *Acta Physiol Scand* 1960 49 Suppl. 173

The high affinity binder may represent a specific carrier protein for folate in serum. Preliminary studies indicate that a binder with similar characteristics is present in male serum. Furthermore, folate binding in milk (Hansen *et al* 1977; Hansen *et al* 1978) displays features similar to those of specific folate binding in serum.

References

- HANSEN, S. L. J. HOLM and J. LYNGBY, *Scand. J. clin. Lab. Inv.* 1977 37 363-367
HANSEN, S. L. J. HOLM and J. LYNGBY, *Biochim. Biophys. Acta* 1978 533 309-318

D 30

Fate of Receptor Bound Glucagon in Adipocytes

By OLE SONNE and JØRGEN GLIEMANN *Institute of Medical Physiology C The Panum Institute University of Copenhagen Denmark*

The interaction of polypeptide hormones with their receptors (defined as saturable binding sites) has generally been assumed to be a bimolecular reversible reaction $H + R \rightleftharpoons RH$. However, in the case of insulin binding to rat adipocytes we have shown (Gliemann & Sonne, 1978) that about half of the ^{125}I -labelled hormone bound to receptors at steady state is degraded and that the radioactivity dissociates from the cells as iodotyrosine.

Receptor-mediated breakdown of polypeptide hormones might be a more general phenomenon and we have therefore studied the fate of receptor bound ^{125}I -glucagon. In

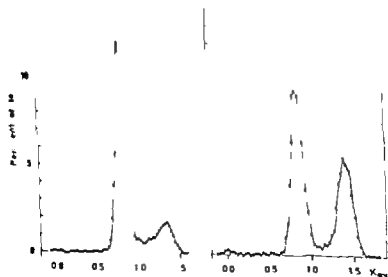


Fig. 1 For explanation see text

High and Low Affinity Binding of Folate in Human Serum

By J. HOLM, S. I. HANSEN and J. LYNGBYE. *Department of Clinical Chemistry, Central Hospital Hillerød, Denmark*

The high folate binding activity normally observed in serum during pregnancy prompted the present study on binding of ^3H folate in sera from pregnant women. Scatchard analyses of data from Equilibrium dialysis experiments revealed two classes of binding sites: a high affinity type ($K(\text{equilibrium constant}) = 10^8 \text{ M}^{-1}$, $N(\text{maximum bound folate}) = 0.4 \text{ nM}$) and a low affinity type ($K = 10^4 \text{ M}^{-1}$). The high affinity folate binder was eluted from sera subjected to anion exchange chromatography (DEAE Sepharose CL) 6B (imidazole buffer of pH 6.3 with 30 mM NaCl). The protein was present in tracer amounts and had a molecular weight of 25 000 as determined by Ultrogel AcA-44 gel filtration. The low affinity binder ($M = 70\,000$) which was eluted from the DEAE Sepharose column following a rise in NaCl to 1 M was identical to albumin.

High affinity binding of ^3H folate displays positive cooperativity as indicated by the downward concavity of the Scatchard plot (Fig. 1). Half saturation of binding was achieved at a folate concentration of 10^{-1} M and saturation at 10^{-8} M . The folate analogue methotrexate is a rather weak inhibitor of folate binding (50% inhibition at a molar methotrexate:folate ratio of 100). Inhibition seemed to be of a competitive type since no decrease in maximum bound folate was seen in presence of methotrexate. Dissociation of ^3H folate from the high affinity binder was slow and incomplete at pH 7.4 but became rapid and complete at pH 3.5.

Low affinity binding of ^3H folate was proportional to the folate concentration within the interval 10^{-10} M to 10^{-7} M . Methotrexate had no effect on this binding which was loose and dissociable.

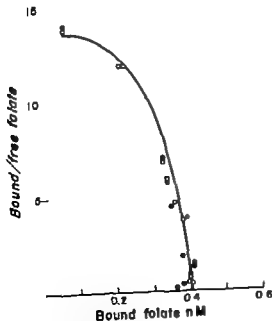


Fig. 1. Scatchard plot of folate binding to a high affinity binder in sera from pregnant women. The binder was eluted from sera subjected to DEAE-Sephacose CL-6B ion exchange chromatography (for details of test). Equilibrium dialysis experiments at 37°C in Tris buffer of pH 7.4 (single determinations). Abcissa: bound folate. Ordinate: bound/free folate.

The high affinity binder may represent a specific carrier protein for folate in serum. Preliminary studies indicate that a binder with similar characteristics is present in milk serum. Furthermore, folate binding in milk (Hansen *et al* 1977, Hansen *et al* 1978) displays features similar to those of specific folate binding in serum.

References

- HANSEN, S. I., J. HOLM and J. LYNGBY, *Scand J clin Lab Invest* 1977 37 363-367
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D 30

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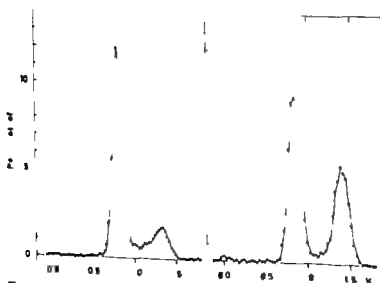


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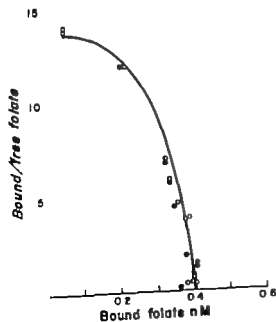


Fig. 1 Scatchard plot of folate binding to a high affinity binder in sera from pregnant women. The binder was eluted from sera subjected to DEAE-Sepharose CL-6B ion exchange chromatography (for details cf. text). Equilibrium dialysis experiments at 37°C in Tris buffer of pH 7.4 (single determinations). Abscissa: bound folate. Ordinate: bound/free folate.

The cerebral unidirectional extraction of glucose (E) was measured 5 s after a single stereoid isotonic injection of a flow-limited reference substance and labeled D-glucose according to Oldendorf's method (1970) with or without the presence of 1 mM buffered ammonium acetate in the injectate. In sham-operated rats the presence of ammonia in the injectate caused an increase of E from 13.1% to 18.3% ($P < 0.05$). No change of F was observed in rats with PCA for 4 weeks. In rats with PCA for 8 weeks, the presence of ammonia in the injectate caused a decrease of E from 13.2% to 9.0% ($P < 0.02$). Blood ammonia was increased from the control value of 100 μ M to 400 μ M in both groups of PCA-rats.

We conclude that high ammonia levels at the blood-brain barrier reduce the efficiency of the glucose carrier in proportion to the length of exposure to moderately elevated blood ammonia concentrations, as seen in long-standing portal-systemic hyperammonemia.

References

- FERRY, E. A. and E. A. BORTON *Neurology* 1969 19 297-298.
 GIBBO, A. A. H. LOCKWOOD, T. E. DUFFY and F. PLUM *Ann Neurol* 1978 3 325-330.
 OLDENDORF, W. H., *Brain Research* 1970 24 372-376.

D 32

Modulation of Pinocytosis by Sulfhydryl (SH) Compounds

By P. JOHANSSON and J.-O. JOSEFSSON *Department of Pharmacology University of Lund S. ed. A*

The intensity of pinocytosis in *Amoeba proteus* measured by the number of pinocytotic channels is sensitive to several physical and chemical stimuli. Cation induced pinocytosis is inhibited by competing non-inducing cations (e.g. Ca^{2+}) and by agents interfering with the biochemistry of mechanisms of the pinocytotic process (e.g. inhibitors of metabolism and protein synthesis). Ca^{2+} -binding agents and drugs which stabilize membranes or affect microtubules. By increasing the free intracellular Ca^{2+} concentration (Ca_i^{2+}) in the cell to a level above the optimum for channel formation some hormones and compounds (e.g. dbcAMP, the Ca^{2+} -ionophore A 23187 and caffeine) inhibit pinocytosis in normal cells and activate pinocytosis in Ca^{2+} -deficient cells. In contrast to these activating agents a few compounds (resulin, somatostatin, cGMP, morphine and met-enkephalin) oppose blockade of pinocytosis caused by Ca_i^{2+} (Johansson and Josefsson 1978b).

Pretreatment of amoebae with 10^{-3} M L-cysteine for 30 mins at pH 5 inhibits the cycle of pinocytosis induced by 100 mM NaCl at pH 6. The effect is potentiated by dbcAMP (10^{-5} M) or caffeine (10^{-3} M) but reversed by addition of insulin (40 μ E/ml), somatostatin (10^{-6} g/ml), cGMP (10^{-6} M), morphine (10^{-6} M) or met-enkephalin (10^{-6} g/ml).

In the presence of cysteine intensive pinocytosis can be induced by Na⁺ in Ca^{2+} -deficient cell only while in normal cells channels will develop only when a calcium-binding agent is added to the inducer. Both verapamil (10^{-5} M) and procaine (or procainamide

suspended rat adipocytes glucagon is bound with an apparent K_d of $1-2$ nM and half time of dissociation of label is about 5 min (Sonne & Gliemann 1977). Glucagon in the suspension is degraded by proteases (unrelated to receptors) and the rate of degradation by this process is much higher than the total turnover of glucagon on the receptors (Sonne & Gliemann 1977). The following experiments were carried out in order to see whether a receptor mediated degradation existed in addition to the protease mediated 125 I-glucagon (0.1 nM) was incubated with adipocytes (2.5% (v/v) i.e. $2-3 \times 10^8$ cells/ml) for 30 min to achieve steady state of binding. 0.7% of the labelled glucagon in the suspension was bound to receptors and 0.07% was bound to non saturable sites (i.e. in the presence of 1 μ M unlabelled glucagon). Fig. 1 left panel shows the elution profile on Sephadex G50-Fine of radioactivity extracted from cells incubated to steady state of binding. It appears that about 90% of the bound radioactive material elutes as iodoglucagon. Fig. 1 right panel shows the elution profile of radioactivity released from cells into non radioactive medium containing a large concentration of unlabelled glucagon which blocked protease mediated degradation of labelled glucagon. It appears that about 60% elutes as glucagon whereas 40% elutes as small molecular weight components probably iodotyrosine.

It is concluded that about 40% of the turnover of glucagon on receptors in rat adipocytes is accounted for by a process which involves binding, degradation of the bound hormone and release of degraded fragments.

References

- GLIEMANN J and O SONNE *J Biol Chem* 1978 253 In press
 SONNE O and J GLIEMANN *Biochim Biophys Acta* 1977 499 59-72.

D 31

Ammonia Reduces Cerebral Extraction of Glucose in Advanced Portal-Systemic Hyperammonemia

By H. LAURSEN, N. H. DIEMER and A. GJEDDE *Institutes of Neuropathology and Medical Physiology, Dept. A, Copenhagen University, Denmark.*

Abnormally low brain-to-blood glucose concentrations ratios have been observed in certain forms of metabolic encephalopathy including rats with porta-caval anastomosis (PCA) of long duration. A dysfunction of the transport mechanism that facilitates the diffusion of glucose from blood to brain was suggested in these cases and Fishman and Borton (1969) actually observed inhibition of the hexose membrane carrier in many organs including brain in ammonia intoxication. Ammonia is generally believed to play a major role in the development of portal-systemic episodic encephalopathy and the sensitivity of the cerebral metabolism to a marked elevation of blood ammonia in PCA rats increases with length of exposure to moderately elevated blood ammonia levels (Gjedde *et al.* 1978). Therefore the effect of ammonia on the transport of glucose from blood to brain was studied in rats with PCA for 4 or 8 weeks.

We think that these specific increases in peroxisomal activity point to a role for peroxisomes during thermogenesis in brown fat. To our knowledge this is the first example of a physiologically stimulated increase in peroxisomal activity. peroxisomal development (in liver) has hitherto only been stimulated pharmacologically by clofibrate treatment.

If this peroxisomal β -oxidation should play a role during thermogenesis the acetyl-CoA produced should be oxidised in the citric acid cycle in the mitochondria. This means that the condensing partner oxaloacetate should be present in sufficient amounts to accept acetyl-CoA of both mitochondrial and peroxisomal origin. The need for anaplerotic reactions is thus obvious.

We have therefore investigated the activity of pyruvate carboxylase in brown fat. We found that this oxaloacetate-forming enzyme was present and had an activity of 40 nmol per min per mg mitochondrial protein and that it in isolated mitochondria could produce sufficient oxaloacetate not to limit the rate of fatty acid combustion. Further we could demonstrate by malonate inhibition of norepinephrine-stimulated respiration in brown adipocytes that its activity led to increased concentrations of citric acid cycle intermediates within the mitochondria.

We have found that pyruvate carboxylation is necessary for maximal thermogenesis to occur even when drainage of citric acid intermediates is eliminated. This means that the anaplerotic reaction is not used for synthetic reactions as is usually the case. Rather the reaction observed is an *internal anaplerotic* one as defined by Scrutton (1975). A similar mechanism has been described for insect flight muscle (Crabtree *et al.* 1972).

These results also explain observations on isolated brown adipocytes where the thermogenesis is stimulated by CO_2 bubbling (Pettersson 1977) and of an increased uptake of glucose into the brown fat *in vivo* during thermogenesis (Portet *et al.* 1974). The glucose can be converted into pyruvate and together with bicarbonate be used for the internal anaplerotic reaction described.

References

- CRABTREE B. S. J. HIGGINS and E. A. NEWSHOLME, *Biochem. J.* 1972 130 391-396.
 LAMON P. B. and C. DEDUVE, *Proc. Natl. Acad. Sci. USA* 1976 73 2043-2046.
 PETTERSSON, B. *Eur. J. Biochem.* 1977 72 235-240.
 PORTET R. M. C. LARRY R. BERTIN C. SEMUEL M. T. HLUZIKO and L. CHEVELLARD, *Proc. Soc. Exp. Biol. Med.* 1974 147 807-812.
 SKITTON M. C. *Med. Hypotheses* 1975 1 207-211.

D 34

Extracellular K^+ Concentration and Regional Blood Flow in Rat Brain Following 10 min of Cerebral Ischemia

By A. J. HANSEN, A. GJEDDE and E. SIEMKOWICZ, Institute of Medical Physiology, Dept. A Pan m Institute and Anesthe la Dept. H idovre Hospital Copenhagen Denmark.

The extracellular potassium concentration, K_{ex} , in brain cortex increases during ischemia (Hansen 1978) but it is not known how K_{ex} changes after an episode of brain ischemia.

10^{-3} M) which reduce the effects of dbcAMP and caffeine respectively diminish cysteine blockade of pinocytosis. Cysteine on the other hand antagonizes the inhibition caused by colchicine (10^{-3} M) and activates the capacity for pinocytosis in starved cells and so imitates dbcAMP and caffeine. Several other thiols and antioxidants viz. glutathione, β -mercaptoethanol, cysteamine, l ascorbate (all 10^{-3} M), α tocopherol (2×10^{-4} M) and butylated hydroxytoluene (3×10^{-4} M) have effects similar to cysteine.

Agents which oxidize or bind thiols e.g. diamide (10^{-3} M), DTNB, oxidized glutathione, oxidized dithiothreitol, sodium nitroprusside (all 10^{-3} M) and CdCl_2 (10^{-4} M) antagonize the effects of cysteine. Diamide inhibits pinocytosis in Ca^{2+} -deficient cells and reverses caffeine blockade of pinocytosis. These effects are antagonized by cysteine and ascorbate.

The results indicate that SH groups are concerned with the central roles of Ca^{2+} and cyclic nucleotides in pinocytosis. Protection of SH groups or reduction of disulphides in the membrane and the cell interior may increase Ca^{2+} and so activate and eventually inhibit channel formation. Oxidation or reduction of critical SH groups within the tissue may therefore be a useful method for modulation of pinocytosis.

References

- JOHANSSON P. and J.-O. JOSEFSSON *Acta physiol. scand.* 1978a 102: 71A-72A.
JOSEFSSON J.-O. and P. JOHANSSON 1978b 7th Int. Congr. Pharmacol. Paris Abstr. 543.

D 33

Thermogenesis in Brown Adipose Tissue: The Possible Production of Acetyl-CoA in Peroxisomes and the Need for Internal Anaplerotic Reactions for Maximal Thermogenesis

By JAN NEDERGAARD, STEFFAN ALEXSON and BARBARA CANNON. *The Wenner-Gren Institute, University of Stockholm*

The substrate for heat production in brown adipose tissue is fatty acids. When the tissue is stimulated by norepinephrine, fatty acids are released from the triglyceride droplets within the tissue, when these fatty acids are combusted, heat is released.

The breakdown of fatty acids to acetyl-CoA has been thought to occur exclusively through mitochondrial β -oxidation. Recently, however, Lazarow and de Duve (1976) found β -oxidation occurring in the peroxisomes in rat liver. We have examined whether peroxisomal β -oxidation could occur in brown adipose tissue.

We found that the peroxisomal cyanide-insensitive NAD-dependent palmitoyl-CoA oxidase was present in brown fat. We have followed this enzyme, as well as the classical peroxisomal enzyme catalase, and the mitochondrial enzyme cytochrome oxidase, during cold-adaptation of rats, and compared the activity of these enzymes in cold-adapted (3 weeks at 5°C) and control (3 weeks at 22°C) rats. Whereas cytochrome oxidase during cold-adaptation increased to 160% of the controls (per g wet weight), palmitoyl-CoA oxidase increased to 230% and catalase to 460%. If the values are related to the total amount of brown fat in the rat, the increases were to 380%, 610% and 950% respectively.

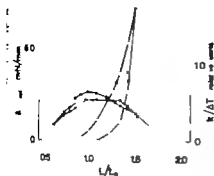


Fig. 1 ○- Relaxed wall tension, T_{rel} (Vessel held in Ca-free solution). ●- Active wall tension, $\Delta T = T_{act} - T_{rel}$, where T_{act} is activated wall tension (vessel held in K⁺-rich solution containing 40 μ M noradrenaline). A Ratio of active dynamic stiffness, k , (difference between dynamic stiffness when activated and when relaxed) to ΔT . Values plotted against relative internal circumference L/L_0 . Vessel was activated for 2 min every 15 min and internal circumference set to given values in the order indicated by arrows while vessel was relaxed. ■ ◆ ΔT and $k/\Delta T$ respectively measured in two activations at end of experiment. $L_0/r = 160 \mu$ m. Segment length = 0.73 mm. Active stiffness at L_0 was 40 $\Delta T/L_0$. Temperature 37°C.

$L/L_0 = 1.64 \pm 0.11$ ($N=5$). Here k was proportional to ΔT . Below L_0 , ΔT fell with decreasing L , but the ratio $k/\Delta T$ increased.

The results suggest that the fall in ΔT above L_0 could indeed be due to a reduction in overlap of contractile filaments, but that below L_0 the fall may in part be due to steric interference between such filaments.

References

- ASHTON T. A. V. SORELLO and A. P. SORELLO *J. Mol. Biol.* 1975 98 17-29
 MILLER M. J. and W. HALPERN, *Nature* 1976 260 617-619

D 36

A Local Denaturation, Residue Detection Method for Simultaneous Measurements of Regional Hepatic Blood Flow and Distribution Volumes for Extracellular Indicators

By WILLIAM P. PAASKE, *Institute of Medical Physiology B, University of Copenhagen, Denmark*

The present study was undertaken in order to determine blood flow and distribution volumes for extracellular hydrophilic indicators in minute areas of the liver.

The experiments were performed in rabbits anaesthetized with sodium pentobarbitone. A median abdominal incision was made in order to expose the liver. The hepatic artery and vein were clamped. 50 μ l isotonic saline containing about 0.5 mCi 51 Cr-ethylene-diamine-tetraacetate (51 Cr-EDTA), was applied to the surface of the liver for 5 min during which time the exposed areas were covered with polyethylene sheets. After this period the sheets were removed and the area was rinsed with isotonic saline. 50 μ l isotonic saline containing about 1 mCi 133 Xenon was then applied to the field which was immediately covered with

Ischemia was induced in halothane anesthetized normoglycemic rats by inflation of a pneumatic cuff placed around the neck and lowering of blood pressure by bleeding (Siemkiewicz and Hansen 1978). After craniotomy of the left parietal bone K_e was measured in the cortex and basal ganglia by means of K^+ -selective microelectrodes. Cerebral blood flow was determined by the method of Schaefer *et al* (1976). A bolus of ^{14}C -butanol was rapidly injected in a femoral vein and arterial blood was sampled continuously for 70 s until heart action was quickly arrested by i.v. injection of saturated KCl. ^{14}C activity in the brain was measured for calculation of regional blood flow.

During ischemia K_e increased identically in the two regions. An initial slow increase to 10 mM for 2 min was followed by a rapid increase to 70 mM and a final slow increase to 80 mM.

After 10 min the cuff was deflated and blood reinfused. Adrenaline was given when necessary in order to maintain blood pressure above 100 mmHg. K_e reached preischemic levels after 4 min in the cortex and after 2 min in the basal ganglia. The decrease of K_e displayed two phases. A slow phase from 80 to 50 mM for 3 min in the cortex and for 1 min in the basal ganglia, and a second rapid decline from 50 to 3 mM lasting 1 min in both regions. After about 1 min the blood flow was 21 ml/100 g/min in the cortex and 155 ml/100 g/min in the basal ganglia but increased to similar high levels in the cortex when the K was normalized.

The experiments thus suggest an intimate relationship between cerebral blood flow and the extracellular potassium concentration after ischemia.

References

- HANSEN A. J. *Acta physiol scand* 1978 102 324-329
 SCHAEFER J. A., A. GJEDDE and F. PLUM *Neurology* 1976 26 394
 SIEMKOWICZ, E. and A. J. HANSEN *Acta neurol scand* 1978 In press

D 35

Mechanical Evidence for a Sliding Filament Mechanism in Vascular Smooth Muscle

By M. J. MULVANY and D. M. WARSHAW *Biophysics Institute Aarhus University Denmark*

The structural evidence (Ashton *et al*, 1975) for a sliding filament mechanism in smooth muscle has been tested by determining the effects of stretch on active isometric wall tension ΔT and active dynamic stiffness k . Rat mesenteric arterial resistance vessel segments were mounted on a myograph (Mulvany & Halpern 1976). In 5 experiments similar to that shown in Fig. 1 ΔT and k were measured in the range 0.6 L_0 to 1.6 L_0 , where L_0 is the internal circumference L_i giving peak active isometric wall tension ΔT_0 . Over this range the changes determined were essentially reversible. Above L_0 ΔT fell almost linearly with increasing L_i , the points lying close to a line which cut the abscissa at

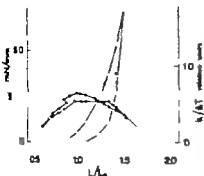


Fig. 1 ○ Relaxed wall tension, T_{rel} (Vessel held in Ca-free solution). ● Active wall tension, T_{act} . $\Delta T = T_{act} - T_{rel}$, where T_{act} is activated wall tension (vessel held in K⁺-rich solution containing 40 μ M noradrenaline). ▲ Ratio of active dynamic stiffness, k , (difference between dynamic stiffness when activated and when relaxed) to ΔT . Values plotted against relative internal circumference L/L_0 . Vessel was activated for 2 min every 15 min and internal circumference set to given values in the order indicated by arrows while vessel was relaxed. ■ ◆ ΔT and $k/\Delta T$ respectively measured in two activations at end of experiment. $L_0/r = 160 \mu$ m. Segment length = 0.73 mm. Active stiffness at L_0 was 40 $\Delta T_0/L_0$. Temperature 37°C

$L/L_0 = 1.64 \pm 0.11$ ($N=5$). Here k was proportional to ΔT . Below L_0 , ΔT fell with decreasing L , but the ratio $k/\Delta T$ increased.

The results suggest that the fall in ΔT above L_0 could indeed be due to a reduction in overlap of contractile filaments, but that below L_0 the fall may in part be due to steric interference between such filaments.

References

- ARON, F. T., A. V. SOMLYO and A. P. SOMLYO *J. Mol. Biol.* 1975 98 17-29.
MILLAR, M. J. and W. HALPERN, *Nature* 1976, 260 617-619.

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A Local Denervation, Residue Detection Method for Simultaneous Measurements of Regional Hepatic Blood Flow and Distribution Volumes for Extracellular Indicators

By WILLIAM P. PAASKE, *Institute of Medical Physiology B University of Copenhagen Denmark*

The present study was undertaken in order to determine blood flow and distribution volumes for extracellular hydrophilic indicators in minute areas of the liver.

The experiments were performed in rabbits anesthetized with sodium pentobarbitone. A median abdominal incision was made in order to expose the liver. The hepatic artery and vein were clamped. 50 μ l isotonic saline containing about 0.5 mCi ^{51}Cr -ethylene-diamine-tetraacetate (^{51}Cr -EDTA), was applied to the surface of the liver for 5 min during which time the exposed areas were covered with polyethylene sheets. After this period the sheets were removed and the area was rinsed with isotonic saline. 50 μ l isotonic saline containing about 1 mCi ^{133}Xe was then applied to the field which was immediately covered with

Ischemia was induced in halothane anesthetized normoglycemic rats by inflation of a pneumatic cuff placed around the neck and lowering of blood pressure by bleeding (Siemkiewicz and Hansen 1978). After craniotomy of the left parietal bone K_e was measured in the cortex and basal ganglia by means of K^+ selective microelectrodes. Cerebral blood flow was determined by the method of Schaefer *et al* (1976). A bolus of ^{14}C -butanol was rapidly injected in a femoral vein and arterial blood was sampled continuously for 20 min until heart action was quickly arrested by i.v. injection of saturated KCl. ^{14}C activity in the brain was measured for calculation of regional blood flow.

During ischemia K_e increased identically in the two regions. An initial slow increase to 10 mM for 2 min was followed by a rapid increase to 70 mM and a final slow increase to 80 mM.

After 10 min the cuff was deflated and blood reinfused. Adrenaline was given when necessary in order to maintain blood pressure above 100 mmHg. K_e reached preischemic levels after 4 min in the cortex and after 2 min in the basal ganglia. The decrease of K_e displayed two phases: A slow phase from 80 to 50 mM for 3 min in the cortex and for 1 min in the basal ganglia, and a second rapid decline from 50 to 3 mM lasting 1 min in both regions. After about 1 min the blood flow was 21 ml/100 g/min in the cortex and 155 ml/100 g/min in the basal ganglia but increased to similar high levels in the cortex when the K_e was normalized.

The experiments thus suggest an intimate relationship between cerebral blood flow and the extracellular potassium concentration after ischemia.

References

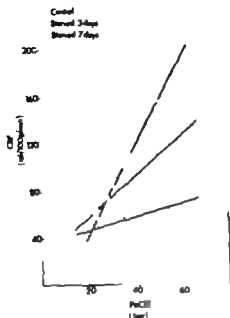
- HANSEN A. J. *Acta physiol scand* 1978 102 3 4-329
 SCHAEFER J. A., A. GJEDDE and F. PLUM *Neurology* 1976 26 394
 SIEMKOWICZ, E. and A. J. HANSEN *Acta neurol scand* 1978 In press

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References

- CARON, J. J. A. GREGOR, D. J. REES and P. PLUM. *Blood Flow and Metabolism in the Brain*. Churchill Livingstone, Edinburgh 1975 pp 3 14-3 17.
 GREGOR, A. B. RINDFELT, J. J. CARON and P. PLUM. *Am. J. Physiol.* 1975 229 114-118.
 HESTED, D. D. and M. L. MARCUS. *Circulation Res.* 1978 42 295-302.
 YANG, J. B. and L. LANDSBERG. *Science* 1977 196 1473-1475.

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Decreased Sympathetic Vasoconstrictor Tone in Diabetic Orthostatic Hypertension

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In normals, subcutaneous blood-flow in the ankle region measured by means of the ^{133}Xe washout technique decreases 60% when the position of the ankle is changed from cardiac level to 50 cm below the heart. A sympathetic axon reflex is responsible for this flow reduction.

The aim was to determine if this reflex control of blood-flow is intact in long-term female diabetics.

A normal response (blood-flow reduction 53 (36-58)% (mean and range)) was found in 6 diabetics without neuropathy age 25 years (mean) duration of diabetes 12 years (mean). In 3 diabetics with autonomic neuropathy (beat-to-beat variation in heart rate < 10/min during

a gas tight mylar membrane and polyethylene sheets. The clamp was removed giving a period of occlusion which had a total duration of about 5.5 min. The washouts of ^51Cr EDTA and $^{133}\text{Xenon}$ were recorded by residue detection using a one inch NaI(Tl) scintillation detector. After cross-talk and background corrections the initial slopes of the washout curves were determined by the "least squares" method. The blood flow was calculated from the equation $f(b) = k_{ex} \lambda_{ex} / 100 \text{ (ml/100 g min)}$ where $f(b)$ is blood flow, k_{ex} is the rate constant of the initial slope (min^{-1}) and λ is the blood to tissue partition coefficient of $^{133}\text{Xenon}$ (0.7 ml/g). The distribution volume of ^51Cr EDTA V_d was calculated from the equation $V_d = f(p) / k$ where $f(p)$ is plasma flow and k is the mean transit time of ^51Cr EDTA. k was taken as the reciprocal value of the rate constant of the initial slope of the ^51Cr EDTA washout. Washout of ^51Cr EDTA was considered to be blood flow limited.

Blood flow was $93.4 \text{ ml/100 g min}$ (S.E. 23.6 , $n=9$). At a hematocrit value of 0.37 (S.E. 0.010) this corresponds to a plasma flow of $53.9 \text{ ml/100 g min}$ (S.E. 15.7). V_d was 23.0 ml/100 g (S.E. 1.5) which is in agreement with the value found by Goresky (1963) in the dog by an independent multiple indicator dilution method using sucrose as indicator.

Reference

- GORESKY C. A. A linear method for determining liver sinusoidal and extravascular volumes. *Amer J Physiol* 1963 204: 626-640.

D 37

Reduced Cerebral Blood Flow Response to Carbon Dioxide in Fasting Rat

By A. GJEDDE and S. M. DE LA MONTE. *Institute of Medical Physiology, Dept. A, Copenhagen University, Denmark.*

The contribution of central and peripheral noradrenergic neurons to the regulation of cerebral blood flow is the subject of dispute (Heistad and Marcus 1978). Reserpine treatment of rats established no role for these neurons in the control of normal cerebral circulation although the response to hypocapnia was reduced during hypotension (Caronna *et al.* 1975).

Starvation in rat causes suppression of the sympathetic nervous system (Young and Landsberg 1977). Therefore we studied the response of the cerebral circulation to acute changes of arterial carbon dioxide tension (Pa_{CO_2}) in rats starved for 3 or 7 days. Whole brain blood flow (CBF) was measured by withdrawal of arterial and cerebral venous blood during washout of xenon in rats anesthetized with halothane and ventilated with varying amounts of CO_2 (Gjedde *et al.* 1975). The result is shown in Figure 1. The $\Delta\text{CBF}/\Delta\text{Pa}_{\text{CO}_2}$ ratio declined from 4% in control rats to 1.6% in rats starved for 3 days and was virtually abolished (0.6%) in rats starved for 7 days.

We conclude that the sympathetic nervous system affects the cerebral circulation under abnormal conditions such as hypotension or starvation.

but the capillary density of the soleus muscle is less than in other extremity muscles (Andersen and Kroese 1978). The tortuous arrangement of the capillaries in the soleus muscle can give this muscle a relatively larger capillary volume. On the other hand the large size muscle fibres of the soleus muscle will result in an on a relative basis smaller surface area. It is then concluded that the difference that exist in extra-cellular water volume between the two muscle studied can only partly be explained by the differences in fibre composition, and factors such as fibre size and capillarization (number and length) which varies between muscles of man may be equally important.

References

- SESTER, F. A. and G. WOO. *Am. J. Physiol.* 1963 206 (4) 1290-1294.
 ANDERSEN, P. and KROESE. *Pflüg Arch.* 1978. In press.
 SUGGARD, G. *Pflüg Arch.* Submitted.

hyperventilation) age 32 years (mean) duration of diabetes 20 years (mean) the reduction in blood-flow upon lowering of the ankle was slightly diminished (blood flow reduction 42 (26-93)%) A marked deterioration of the response was found in 6 diabetics with autonomic neuropathy including orthostatic hypotension in whom blood-flow increased to 108 (65-164)% age 39 years (mean) duration of diabetes 23 years (mean) Ankle blood-flow at cardiac level was similar in the three groups

In conclusion in patients with diabetic orthostatic hypotension the subcutaneous axon reflex is absent which may be one of several factors responsible for their orthostatic symptoms

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Inulin Space In Red and White Skeletal Muscle of Man

By GISELA SJØGAARD and BENGT SALTIN *August Krogh Institute University of Copenhagen Denmark*

In species like the rat two main skeletal muscle fibres types red and white can be found with distinct differences in their metabolic profiles Further differences in intra- and extra-cellular water and electrolyte distribution have been demonstrated (Sreter and Woo 1963)

Also in man two main fibre types can be found the soleus muscle being predominantly composed of "red" fibres while the triceps brachii muscle is predominantly white These muscles were sampled from 6 healthy young males and 6 healthy young females Muscle fibre type composition was based on the staining for myofibrillar ATPase "red" = slow (ST) and "white" = fast (FT) The shortest diameter was used for calculation of fibre area. The total water was obtained by weighing the muscle sample before and after drying Extra-cellular volume (H_2O_e) was measured as inulin distribution space in the supine position using an intravenous dose of 1 mCi tritiated inulin and with blood and muscle sampling after 1 hour (Sjøgaard submitted)

All subjects had higher % ST fibres in the soleus than in the triceps muscle mean values (range) being 73 (51-90)% ST and 39 (19-60)% ST respectively ($p < 0.05$) Mean fibre diameter was larger in the soleus muscle giving approximately 50% larger fibre areas in the soleus as compared to the triceps muscle Total muscle water averaged 371 (399-337) and 318 (295-333) ml/100 g dry weight for the soleus and triceps muscles respectively ($p > 0.05$) The extra-cellular space however was larger in the soleus than in the triceps muscle mean values (range) 55 (38-77) and 42 (18-62) ml/100 g dry weight respectively ($p < 0.05$) None of the variables were significantly different between males and females

There was a relationship between % ST fibres and H_2O_e but it was equally evident that the data points for the two muscles were grouped separately which means that at the same per cent ST fibre H_2O_e was lowest in the triceps muscle

As an explanation for the present finding factors like capillarization and fibre sizes of the two muscles may contribute There are slightly more capillaries around ST than FT fibres

